IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: David WALLACH et al

Application No.: 09/824,134 Conf. No. 2547

Filed: April 3, 2001

For: MODULATORS OF THE FUNCTION OF FAS/APO1 RECEPTORS

Art Unit: 1642

Examiner: M. Davis

Washington, D.C.

Atty.'s Docket: WALLACH=16A

Date: March 6, 2006

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Alexandria, VA 22314

Sir:

Transmitted herewith is a [ ] Amendment [X] Appeal Brief in the above-identified application.

[ ] Small Entity Status: Applicant(s) claim small entity status. See 37 C.F.R. §1.27.

[ ] No additional fee is required.

[XX] The fee has been calculated as shown below:

	(Col. 1) CLAIMS REMAINING AFTER AMENDMENT		(Col. 2) HIGHEST NO. PREVIOUSLY PAID FOR	(Col. 3) PRESENT EXTRA EQUALS	SMALL ENTITY RATE	SMALL ENTITY ADDITIONAL FEE	OR	OTHER THAN SMALL ENTITY RATE	OTHER THAN SMALL ENTITY ADDITIONAL FEE
TOTAL	* 9	MINUS	** 20	0	x 25	$		x 50	$
INDEP.	* 1	MINUS	*** 3	0	x 100	$		x 200	$
FIRST PRESENTATION OF MULTIPLE DEP. CLAIM					+ 180	$		+ 360	$
					ADDITIONAL FEE TOTAL	$	OR	TOTAL	$

* If the entry in Col. 1 is less than the entry in Col. 2, write "0" in Col. 3.

** If the "Highest Number Previously Paid for" IN THIS SPACE is less than 20, write "20" in this space.

*** If the "Highest Number Previously Paid for" IN THIS SPACE is less than 3, write "3" in this space.

The "Highest Number Previously Paid For" (total or independent) is the highest number found from the equivalent box in Col. 1 of a prior amendment of the number of claims originally filed.

[XX] Conditional Petition for Extension of Time

If any extension of time for a response is required, applicant requests that this be considered a petition therefor.

[ ] It is hereby petitioned for an extension of time in accordance with 37 CFR 1.136(a). The appropriate fee required by 37 CFR 1.17 is calculated as shown below:

Small Entity		Other Than Small Entity	
Response Filed Within		Response Filed Within	
[ ] First	- $ 60.00	[ ] First	- $ 120.00
[ ] Second	- $ 225.00	[ ] Second	- $ 450.00
[ ] Third	- $ 510.00	[XX] Third	- $ 1020.00
[ ] Fourth	- $ 795.00	[ ] Fourth	- $ 1590.00
Month After Time Period Set		Month After Time Period Set	

[ ] Less fees ($_____) already paid for ___ month(s) extension of time on _____.

[ ] Please charge my Deposit Account No. 02-4035 in the amount of $_____.

[XX] Credit Card Payment Form, PTO-2038, is attached, authorizing payment in the amount of $ 1,020.00.

[ ] A check in the amount of $_____ is attached (check no.).

[XX] The Commissioner is hereby authorized and requested to charge any additional fees which may be required in connection with this application or credit any overpayment to Deposit Account No. 02-4035. This authorization and request is not limited to payment of all fees associated with this communication, including any Extension of Time fee, not covered by check or specific authorization, but is also intended to include all fees for the presentation of extra claims under 37 CFR §1.16 and all patent processing fees under 37 CFR §1.17 throughout the prosecution of the case. This blanket authorization does not include patent issue fees under 37 CFR §1.18.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:
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MARK BOLDIN, EUGENE VARFOLOMEEV, and
IGOR METT
Application No. 09/824,134
Filed: April 3, 2001

MODULATORS OF THE FUNCTION OF FAS/AP01 RECEPTORS

Examiner: Minh Tam B Davis
Art Unit: 1642

APPEAL BRIEF

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REAL PARTY IN INTEREST

The present application is owned by Yeda Research and Development Co. Ltd., which is the research and development arm of the Weizmann Institute of Science in Rehovot, Israel. The exclusive licensee of the present invention is Inter-Lab Limited, an Israeli company of Ness-Ziona, Israel. Inter-Lab Limited is a subsidiary of InterPharm Laboratories Limited, an Israeli company of Ness-Ziona, Israel, which is a member of the Serono group of companies, whose parent company is Serono S.A., a holding company under which there are many subsidiaries worldwide.

RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences.

STATUS OF CLAIMS

Claims 1-7, 11 and 14 are pending in the present application and are subject to the present appeal. Claims 8-10, 12 and 13 have been cancelled.

STATUS OF AMENDMENTS

No amendment has been made subsequent to the most recent rejection of June 6, 2005, in this case. The Official action of June 6, 2005, was not a final rejection, although it is the fifth Official action on the merits issued in this case by the same examiner (without any intervening Request for Continued Examination). The last final rejection was dated March 4, 2004, and the claims have not been amended since that date.

SUMMARY OF CLAIMED SUBJECT MATTER

The only independent claim in this case is claim 1. No means plus function or step plus function as permitted by 35 U.S.C. §112, sixth paragraph, are present in claim 1.

Claim 1 is directed to an isolated DNA molecule as defined by any one of three alternative numbered paragraphs. Paragraph (1) of claim 1 is directed to the isolated DNA molecule comprising a DNA sequence that encodes the MORT-1 protein, having the amino acid sequence of SEQ ID NO:2. Thus, this portion of the claim is directed to any isolated DNA molecule that includes within it any DNA sequence that encodes the amino acid sequence of SEQ ID NO:2. The amino acid sequence of SEQ ID NO:2 is shown in Fig. 4. The protein having the amino acid sequence of SEQ ID NO:2 is defined in the specification as HF1 (see line 3 of the amended paragraph beginning at page 14, line 20, of the present specification). This novel protein is also known as MORT-1 (for "Mediator Of Receptor Toxicity"). See page 7, lines 19-20.

This paragraph of claim 1 covers not only the DNA sequence of the natural cDNA that encodes MORT-1, but also those sequences that are degenerate as a result of the genetic code to the cDNA sequence derived from the coding region of the native MORT-1 protein (see page 8, line 13-14). The claimed DNA sequence in paragraph 1 may be longer than the

sequence that encodes the MORT-1 protein so as to encompass, for example, vectors that contain the DNA sequence of the invention, but which also contain additional DNA regions that allow them to be capable of being expressed in suitable eukaryotic or prokaryotic host cells (see page 8, lines 24-27). Note that the sentence bridging pages 16 and 17 indicates that the MORT-1 protein may be conjugated to another molecule, for example an antibody, enzyme, receptor, etc., as are well known in the art. Note also the first paragraph on page 30, which refers to recombinant animal virus vectors encoding the MORT-1 protein, but which also encode a virus surface protein.

In paragraph (2) of claim 1, there is claimed an isolated DNA molecule comprising a DNA sequence that encodes an analog of the MORT-1 protein having the amino acid sequence of SEQ ID NO:2. This analog must bind with the intracellular domain of the FAS ligand receptor (FAS-IC). Furthermore, the DNA sequence which encodes that analog must be capable of hybridizing to the cDNA encoding SEQ ID NO:2 under moderately stringent conditions. See page 8, lines 10-12. Note that while the isolated DNA molecule "comprises" the DNA sequence that encodes the analog of the MORT-1 protein, it is only the DNA sequence which encodes the analog that must be capable of

hybridization to the cDNA encoding SEQ ID NO:2 under moderately stringent conditions.

The specification teaches that such analogs may be prepared by standard procedures, citing Sambrook et al, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989). See page 16, lines 5-6, and page 47, lines 1-2. In such procedures, the DNA sequences encoding the MORT-1 protein have one or more codons deleted, added or substituted by another to yield analogs having at least a one amino acid residue change with respect to the native protein (see page 16, lines 7-9). Acceptable analogs are those that retain at least the capability of binding to the intracellular domain of the FAS-R (see page 16, lines 9-10).

In paragraph (3) of claim 1, there is claimed an isolated DNA molecule comprising a DNA coding sequence consisting of a DNA sequence that encodes a fragment of the MORT-1 protein that binds with FAS-IC. The MORT-1 protein is that of the amino acid sequence of SEQ ID NO:2. See page 16, lines 20-28 of the present specification. Note that while the isolated DNA molecule "comprises" the DNA sequence that encodes a fragment of the MORT-1 protein, it is only the DNA sequence that encodes the fragment that must be capable of binding with FAS-IC.

GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

First, it should be noted for the record that the prosecution of this case by the examiner (and it has been the same examiner throughout) has been extremely prejudicial to applicants as the examiner has resorted to piecemeal prosecution, which is not permitted by the Rules (see 37 C.F.R. §1.104). The first official action on the merits in this case was on October 23, 2002, and included rejections under 35 U.S.C. §112, second paragraph; 35 U.S.C. §112, first paragraph, written description; 35 U.S.C. §112, first paragraph, enablement; 35 U.S.C. §112, first paragraph, "scope;" 35 U.S.C. §102; and 35 U.S.C. §103. After a detailed response, a new non-final rejection was issued on July 3, 2003, inserting a new rejection under 35 U.S.C. §101 and maintaining only the 35 U.S.C. §112, first paragraph, written description, rejection and making a new rejection under 35 U.S.C. §112, first paragraph, enablement. The other rejections were apparently withdrawn in view of applicants' arguments.

After a further detailed response by applicants, the examiner issued a final rejection on March 4, 2004, objecting only to the second paragraph of claim 1 under 35 U.S.C. §112, first paragraph, enablement. After that, an Appeal Brief was filed, after which a new non-final rejection was issued on

February 25, 2005, containing only a double-patenting rejection. The previous enablement rejection was not repeated. Thus, applicants were under the impression that, if the double-patenting rejection were overcome, the case would be in condition for allowance. However, on June 6, 2005, a fifth non-final Official action was issued by the examiner re-inserting the 35 U.S.C. §112, second paragraph, rejection from the official action of October 23, 2002; re-inserting the 35 U.S.C. §112, first paragraph, written description rejection, from the official action of July 3, 2003; and re-inserting the 35 U.S.C. §112, first paragraph, rejection from the official action of March 4, 2004, all of which had been previously withdrawn by the examiner. While this inappropriate piecemeal prosecution by the examiner is not appealable, we feel it important enough to bring to the Board's attention as it may affect the weight to be given to the examiner's case.

In the non-final rejection of June 6, 2005 (the fifth Official action on the merits in this case), claims 1-7, 11 and 14 were rejected under 35 U.S.C. §112, second paragraph, as being indefinite in the language "moderately stringent conditions." In the Official of June 6, 2005, beginning at page 2, the examiner stated:

> In the response of 02/24/03, Applicant argues that US 5,026,636 defines moderate stringency as conditions that allow detection of sequences at least 75%

> homologous to the probe and that the conditions could be readily determined using a reference text for guidance. Applicant argues that claims of US 4,968,607 include the term "moderate stringency". Applicant recites Ausubel et al, 1987-1 998, Current protocols in Molecular Biol, which teaches how to determine moderate stringency wash conditions, by calculating the decrease in temperature required using the correlation for decrease in Tm percent mismatch. Applicant argues that based on the teaching in the art, the scope of "moderate stringency" could be determined.
>
> Applicant's arguments set forth in paper of 07/03/03 have been considered but are not deemed to be persuasive for the following reasons:
>
> Since there is no definition of moderately stringent hybridization conditions is found [sic] in the specification, one cannot determine the metes and bound of the claimed invention.
>
> Although some US patents use the language or define the language, or some references teach how to determine moderate stringency wash, the definition by another US patent would be just one of possible numerous reasonable interpretations of the claimed moderately stringent hybridization conditions, in view of the lack of definition of the term in the claimed application, and in view that moderate is a relative term. Concerning how to determine moderate stringency wash, this is not an enablement rejection.

In the non-final rejection of June 6, 2005, claims 1-7, 11 and 14 were rejected under 35 U.S.C. §112, first paragraph, written description, with respect to lack of a clear written description of the claimed DNA sequence encoding

an "analog" of the MORT-1 protein having the amino acid sequence of SEQ ID NO:2. In the Official of June 6, 2005, beginning at page 3, the examiner stated:

> Applicant argues in paper of 12/03/03 that binding to the intracellular domain of the FAS ligand receptor is both a physical property and a function. Applicant argues that binding alone is sufficient to establish the function of, for example, serving in affinity chromatography to isolate MORT-1 (p.7, last line of the first paragraph, in the response of 12/03/03). Applicant argues that the function of the protein or polypeptide encoded by the DNA of claim 1 may be any function disclosed in the specification. Applicant argues that it is acceptable to show that one is in possession of a compound by identifying characteristics which includes physical properties.
>
> Applicant recites the Written description guidelines which states that:
>
> > An applicant may also show that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics which provide evidence that applicant was in possession of the claimed invention, i.e., complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics.
>
> Applicant argues that the analog of claim 1(2) is defined by a complete or partial structure and other physical and/or chemical properties. Applicant argues that there is a partial structure because the DNA encoding it must be capable of hybridizing to the cDNA encoding SEQ ID NO:2 under moderately stringent conditions. Applicant argues that this combination of partial structure and

physical and/or chemical [sic] is sufficient to that Applicant was in possession of the claimed invention.

Applicant's arguments set forth in paper of 07/03/03 have been considered but are not deemed to be persuasive for the following reasons:

Contrary to Applicant's arguments, no one of skill in the art would reasonably conclude that Applicant had possession of the claimed DNA sequences encoding a genus of analogs of MORT-1 protein of SEQ ID NO:2 at the time of filing for the reasons of record. The claimed analog lacks definitive function which is correlated with a specific function.

Contrary to Applicant's arguments, binding to FAS-IC alone is not a definitive function, such that the function defines MORT-1. There is no teaching in the specification that would permit one of skill to predictably identify the species included in the claimed genus, and to distinguish between that which is claimed from that which is not claimed. Thus the specification does not provide a written description of the claimed invention, that would demonstrate that Applicant was in possession of the claimed invention at the time of filing.

In addition, concerning Applicant's assertion that function of the claimed analog of serving in affinity chromatography to isolate MORT-1, the encoded sequence consisting of amino acids 130-245 of SEQ ID NO:2 binds to FAS-IC, but does not bind to the MORT-1 protein of SEQ ID NO:2 (specification, p.36, lines 4-5), and thus cannot be used for making affinity column to isolate MORT-1 protein.

Further, there is no correlation provided between the properties of "DNA sequences encoding sequences that bind to FAS-IC" and structure of "a DNA sequence that has the

ability to hybridize to the cDNA encoding SEQ ID NO:2, under moderately stringent conditions", because there are proteins that bind to FAS-IC, such as FAS ligand, and antibodies to FAS-IC, but having a structure completely different from the disclosed amino acids 130-245 of MORT-I of SEQ ID NO:2.

In other words, not only binding to FAS-IC is not a definitive function, there is no correlation between the properties of binding to FAS-IC and structure of a DNA sequence that has the ability to hybridize to the cDNA encoding SEQ ID NO:2, under moderately stringent conditions. The specification discloses only a single cDNA sequence encoding the MORT-1 protein of SEQ ID NO:2, wherein the amino acids 130-245 of SEQ ID NO:2 bind to the intracellular domain of the FAS-ligand receptor. This is insufficient to establish a correlation between the property of binding to FAS-IC, which is shared by unrelated sequences, and structure of a DNA sequence that has the ability to hybridize to the cDNA encoding SEQ ID NO:2.

In addition, although a partial structure is implied in claim 1, by the recitation of a DNA sequence capable of hybridizing to the cDNA encoding SEQ ID NO:2 under moderately stringent conditions, said structure is undefined, and is not correlated with the ability to bind to FAS-IC, because any unrelated DNA sequence with unknown function could hybridize to a CDNA sequence encoding SEQ ID NO:2 under moderately stringent conditions, via a common fragment, wherein said fragment does not necessarily have to encode amino acids 130-245 of the MORT-1 protein of SEQ ID NO:2, a fragment necessary for binding to FAS receptor (specification, page 36). The language "DNA sequence capable of hybridizing to the cDNA encoding SEQ ID NO:2 under moderately stringent conditions" per se does not define that the sequence encodes the amino acids 130-245 of

> the MORT-1 protein of SEQ ID NO:2, a sequence necessary for binding to FAS-IC.
>
> Thus, there is no correlation between structure and function for the claimed DNA sequence encoding an analog of MORT-1 protein of SEQ ID NO:2 in claim 1, that would allow one to distinguish between that which is claimed from that which is not claimed.
>
> Moreover, the specification does not disclose a representative number of species of DNA sequences encoding an analog of MORT-1 protein, wherein which analog binds to FAS-IC, and wherein which DNA sequence is capable of hybridizing to the cDNA encoding SEQ ID NO:2. The specification only discloses a single DNA sequence that encodes the amino acids 130-245 of the MORT-1 protein of SEQ ID NO:2, wherein said amino acid sequence is necessary for binding to FAS-IC.
>
> For the reasons set forth previously and above, the claimed invention does not meet 112, first paragraph, written description requirement, and one would reasonably conclude that Applicant did not have possession of the claimed DNA sequences encoding a genus of analog of SEQ ID NO:2 at the time of filing.

In the non-final rejection of June 6, 2005, claims 1-7, 11 and 14 were rejected under 35 U.S.C. §112, first paragraph, "scope," in that the specification lacks enablement for a DNA sequence encoding a fragment of MORT-1 protein that binds FAS-IC or encoding an "analog" of the MORT-1 protein having the amino acid sequence of SEQ ID NO:2. In the Official of June 6, 2005, beginning at page 7, the examiner stated:

In the brief of 03/04/04 Applicant argues that the Examiner states that Applicant has not taught how to make variants that are capable of hybridizing to the cDNA encoding SEQ ID NO:2 under moderately stringent conditions, such that those variants would bind to the intracellular domain of the FAS ligand receptor, and that determining which analogs binds FAS-IC would not entail undue experimentation, in view of Wands analysis (p.15-20). Applicant argues that mutation method is routine in the art, and that screening is routine, and thus they do not amount to undue experimentation.

Applicant's arguments set forth in paper of 03/04/04 have been considered but are not deemed to be persuasive for the following reasons:

It is noted that the Examiner did not state that Applicant has not taught how to make variants that are capable of hybridizing to the cDNA encoding SEQ ID NO:2 under moderately stringent conditions, **such that those variants would bind to the intracellular domain of the FAS ligand receptor.**

Rather, the Examiner position is that Applicant has not taught how to make variants that are capable of hybridizing to the cDNA encoding SEQ ID NO:2 under moderately stringent conditions, **such that those variants would have the properties and function of the claimed polynucleotides encoding SEQ ID NO:2** (previous Office action of 03/04/04, page 3, fourth paragraph).

The specification discloses that MORT-1 binds to intracellular domain of FAS receptor, and thus is capable of modulating the function of the FFAS [sic] receptor, and that amino acids 1-117 are necessary for self association, and amino acids 130-245 are necessary for binding to FAS receptor (p. 1, first full paragraph, p.36). The specification further discloses that FAS

receptor mediates cell death, and that monoclonal antibodies to FAS receptor could induce apoptotic cell death (p.3, second paragraph).

The specification also discloses that however, MORT-1 can activates [sic] cell cytotoxicity on its own (p. 1, first full paragraph). No disclosure concerning which fragment is responsible for activation of cell cytotoxicity is found in the specification.

It is noted that binding to a receptor does not necessarily mean that the receptor would be activated, because although there is certain plasticity in ligand-receptor interactions, the ligand has to have a certain binding stability, and has to have molecular configuration specificity, for example, a certain configuration for perfect fit into the receptor for activation of the receptor, like lock and key. For example, Zhang et al, 2005, Acta Pharmacoligica Sinica, 26(2): 171-1 76 teach that the effect of an estrogen ligand requires molecular configuration specificity of the ligand. Yoshikawa Noritada et al, Molecular endocrinology, May 2005, 19 (5):pl110-24, teach that for activation of the glucocorticoid receptor, stable conformational changes of the ligand binding domain, induced by binding of the receptor to the ligand seem to be necessary for activation of the receptor. Wilson I A et al, Current opinion in structural biology (ENGLAND) Dec 1999, 9 (6):p696-704, teach that erythropoietin receptor activation is dependent on the actual configuration of the receptor-ligand dimer assembly. Petry Renate et al, Journal of medicinal chemistry (United States) Feb 28 2002, 45 (5): p1026-34, teach that stabilization of a particular structural state of the receptor, and further induction of conformational rearrangements of the receptor by the ligand is necessary for receptor activation.

In view of the art, although one could screen for fragments of SEQ ID NO:2 that bind to FAS-IC, one cannot predict that amino acids 130-245 of SEQ ID NO:2, and variants thereof would activate the FAS receptor. For example, one cannot predict whether amino acids 130-245 of SEQ ID NO:2 would have the molecular configuration specificity required for FAS receptor activation, or would stabilize a certain particular structural state of the FAS receptor or would induce certain conformational changes of the FAS receptor, that might be required for FAS receptor activation.

Thus **one would not know how to use the claimed DNA sequence encoding such fragments that bind to FAS-IC, nor MORT-1 analogs as claimed in claim 1,** and it would be undue experimentation for one of skill in the art to practice the claimed invention.

Further, the claimed analogs would not necessarily have the function and properties of polypeptide encoding SEQ ID NO:2, such as activation of cell cytotoxicity, because the specification does not disclose which fragments of SEQ ID NO:2 are responsible for such activation of cell cytotoxicity. **One would not know how to make the claimed analogs, such that they would have the function of the polypeptide encoding SEQ ID NO:2,** in view of the unpredictability of protein chemistry, as taught by Burgess et al, Lazar et al, Tao et al, and Gillies et al, all of record, and in view that such unpredictability would apply as well to DNA sequences which encode proteins.

It is noted that MPEP 2164.03 teaches that "the amount of guidance or direction needed to enable the invention is inversely related to the amount of knowledge in the state of the art as well as the predictability of the art. In re Fisher, 427 F.2d 833, 839, 166 USPQ 18,24 (CCPA 1970). The amount of guidance or direction refers to that

information in the application, as originally filed, that teaches exactly how to make or use the invention. The more that is known in the prior art about the nature of the invention, how to make, and how to use the invention, and the more predictable the art is, the less information needs to explicitly stated in the specification. In contrast, if little is known in the prior art about the nature of the invention and the art is unpredictable, the specification would need more detail as how to make and use the invention in order to be enabling."

Given the unpredictability that the encoded fragment that binds FAS-IC, or the encoded analogs of MORT-1 protein would activate FAS receptor, and the unpredictability of protein chemistry, which applies as well to DNA sequences encoding proteins, the lack of adequate disclosure in the specification, and in view of the complex nature of the claimed invention, and little is known in the art about the claimed invention, one of skill in the art would be forced into undue experimentation to practice the claimed invention.

ARGUMENT

The Language "Under Moderately Stringent Conditions" Is Not Unduly Vague and Indefinite

The language "under moderately stringent conditions" is not unduly vague and indefinite. MPEP §2173.02 states:

> The examiner's focus during examination of claims for compliance with the requirement for definiteness of 35 U.S.C. 112, second paragraph, is whether the claim meets the threshold requirements of clarity and precision, not whether more suitable language or modes of expression are available. ...
>
> The essential inquiry pertaining to this requirement is whether the claims set out and circumscribe a particular subject matter with a reasonable degree of clarity and particularity. Definiteness of claim language must be analyzed, not in a vacuum, but in light of:
>
> (A) the content of the particular application disclosure;
>
> (B) the teachings of the prior art; and
>
> (C) the claim interpretation that would be given by one possessing the ordinary level of skill in the pertinent art at the time the invention was made.

At the time the present invention was made, the metes and bounds of moderate stringency were known to those of skill in the art, even though there may be some variation in the means for providing roughly the same level of stringency either at the hybridization stage or at the wash stage. U.S. patent 5,026,636, relevant pages of which are attached in the Evidence Appendix, was available at the time the invention was

made and defines moderate stringency as conditions that allow detection of nucleotide sequences at least approximately 75% homologous to the probe (column 4, lines 50-65). This patent further teaches that moderately stringent conditions for a particular probe, when seeking a specified degree of homology, may be readily determined by those of skill in the art using, for example, the reference text, Nucleic Acid Hybridisation: A Practical Approach, Hames and Higgins, eds., IRL Press, Washington (1985) or the scientific publication, Wood et al, Proc Natl Acad Sci USA 82:1585-1588 (1985) (copy of which is attached hereto in the Evidence Appendix), for guidance. Chapter 4 of Nucleic Acid Hybridisation: A Practical Approach, on quantitative filter hybridization, a copy of which is attached hereto in the Evidence Appendix, teaches the various factors affecting hybrid stability through a calculation of melting temperature (T_m) of the hybrid using the standard equation (Equation 7 on page 80) and factoring in the percent mismatch (percent identity) that is sought. The calculation of T_m takes into account the molarity of the monovalent cation (i.e., sodium in SSC solution). Accordingly, those of skill in the art would recognize and understand what the metes and bounds of the conditions needed for moderate stringency hybridization to detect a hybrid with a specified percent identity, e.g., 75%.

Indeed, U.S. patents 4,968,607 (50°C, 2 X SSC; column 10, lines 39-40), 5,171,675 (50°C, 2 X SSC; column 6, line 49), 5,198,342 (50°C, 2 X SSC; column 9, lines 54-55), 5,262,522 (50°C, 2 X SSC; column 15, lines 8-9), and 5,237,051 (60°C, 1 X SSC; column 5, lines 24-27), which were available to the art at the time of the present invention, demonstrate that those in the art were able to determine and define moderately stringent conditions based on the knowledge and skill at that time. All have claims that include the term "moderate stringency". Relevant pages of the above-cited U.S. Patents are attached hereto in the Evidence Appendix. According to the Federal Circuit Court of Appeals, it is relevant to the issue of definiteness that the criticized words are used frequently in patent claims. See *Andrew Corp. v. Gabriel Electronics*, 847 F.2d 819, 6 USPQ2d 2010, 2012-13 (Fed. Cir. 1988). And see also *Ex parte Brian*, 118 USPQ 242, 245 (Bd. App. 1958), where it states:

> Since the claims under consideration are similar to those in the patents, we do not feel disposed to reject them and thus upset such a long established practice in the particular art under consideration.

In other words, the very fact that "moderate stringency" claims have been repeatedly allowed in the past is reason to consider them definite. This is true, not only because the use of such claims by many different inventors and allowed by

many different examiners is evidence that the terminology is considered sufficiently definite by the art, but also because a reinterpretation of the definiteness of such claims by the PTO casts a shadow of doubt on previously issued "moderate stringency" claims, even though such claims are entitled to a presumption of validity.

Furthermore, the widely used reference text, Current Protocols in Molecular Biology, eds. Ausubel et al, John Wiley & Sons, Inc., (1987-1998) on page 2.10.11 (Supplement 26, 1994[1]), a copy of which is also attached in the Evidence Appendix, guides those of skill in the art how to use a rational approach at determining "moderate stringency" wash conditions by calculating the decrease in temperature required using the correlation for decrease in T_m per percent mismatch.

Accordingly, in view of the teachings of the prior art, it is urged that the claim interpretation to be given to the term "hybridization under moderately stringent conditions" are those conditions which would permit detection of nucleotide sequences at least approximately 75% homologous. Thus, the scope of the invention sought to be patented can be determined from the language of the claims with a reasonable degree of certainty.

[1] A copy of page 2.6.1 is also attached in the Evidence Appendix that shows that Supplement 26 is dated 1994).

The examiner does not consider the substantial citation of evidence by applicants in this regard to be convincing to show that one of ordinary skill in the art would consider the term "moderately stringent conditions" to be reasonably definite. The examiner states that there is no definition of "moderately stringent hybridization conditions" found in the specification. The examiner concedes that some U.S. patents use the language or define the language, and some references teach how to determine moderate stringency wash. However, the examiner states that the definition by another U.S. patent would be just one of numerous possible reasonable interpretations of the claimed moderately stringent hybridization conditions in view of the lack of definition of the term in the claimed application and in view of the fact that "moderate" is a relative term.

It is noted, however, that the examiner has not cited any reference to show that one of ordinary skill in the art would consider the term to be other than what applicants have established. As stated in *In re Barr*, 444 F.2d 588, 170 USPQ 330 (CCPA 1971), to answer the question of whether the claims fail in particularly pointing out and distinctly claiming the subject matter that appellants regard as their invention, the claims must be construed from the standpoint of a person skilled in the relevant art. See also paragraph (C)

in the above-quoted section of MPEP §2173.02. Claim interpretation during prosecution is explained at MPEP §2111, which states that claims must be given their broadest reasonable interpretation consistent with the interpretation that those skilled in the art would reach. This section of the MPEP cites *In re Cortright*, 175 F.3d 1353, 1358, 49 USPQ2d 1464, 1467 (Fed Cir 1999), which case states:

> Although the PTO must give claims their broadest reasonable interpretation, this interpretation must be consistent with the one that those skilled in the art would reach. ... Prior art references may be "indicative of what all those skilled in the art generally believe a certain term means ... [and] can often help to demonstrate how a disputed term is used by those skilled in the art." *Vigronics Corp. v. Conceptronic, Inc., 90 F.3d 1576, 1584, 39 USPQ2d 1573, 1578-1579 (Fed. Cir. 1996)*. Accordingly, the PTO's interpretation of claim terms should not be so broad that it conflicts with the meaning given to identical terms in other patents from analogous art. Cf. [*In re Morris*, 127 F.3d 1048, 1056, 44 USPQ2d 1023, 1029 (Fed. Cir. 1997)] (approving the board's definition of claim terms consistent with their definitions in CCPA cases).

Thus, it is permissible to consider how similar language is defined and interpreted in other patents.

As to the examiner's complaint that the term "moderate" is a relative term, reference is made to MPEP §2173.05(b), which states:

> The fact that claim language, including terms of degree, may not be precise, does not automatically render the claim

> indefinite under 35 U.S.C. §112, second paragraph. ... Acceptability of the claim language depends on whether one of ordinary skill in the art would understand what is claimed, in light of the specification.
>
> As stated at MPEP §2173.02:
>
> When the examiner is satisfied that patentable subject matter is disclosed, and it is apparent to the examiner that the claims are directed to such patentable subject matter, he or she should allow claims which define the patentable subject matter with a reasonable degree of particularity and distinctness. [emphasis original]

Here, there is no rejection over the prior art. The examiner is satisfied that the subject matter is patentable over the prior art. Accordingly, the language "moderately stringent conditions," considered in light of the evidence presented herein as to how that term would be construed by one of ordinary skill in the art when giving the present specification its broadest reasonable interpretation, should establish that the patentable subject matter is defined with a reasonable degree of particularity and distinctness.

Indeed, these very arguments were found convincing to this same examiner when presented in applicants' amendment of February 24, 2003. Accordingly, the examiner's reasons for re-inserting the rejection, notwithstanding three intervening Official actions that did not re-insert it, should be given very little weight.

Reversal of the examiner and withdrawal of this rejection are therefore respectfully urged.

The Specification Establishes that Applicants Were in Possession of the Claimed Invention

It is apparent that this written description rejection only applies to the second paragraph of claim 1, which uses the term "analog." This part of the claim makes clear that it is the analog that must bind with the intracellular domain of the FAS ligand receptor (FAS-IC). Thus, the claim includes the function of binding to FAS-IC. Further, this paragraph states that the DNA sequence is capable of hybridization to the cDNA encoding SEQ ID NO:2 under moderately stringent conditions. Thus, this part of the paragraph defines the sequence by structure.

The Guidelines for the Examination of Patent Applications under the 35 U.S.C. §112, paragraph 1, "Written Description" Requirement, as set forth in MPEP §2163, states at II.A.3.(a):

> An applicant may also show that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics which provide evidence that applicant was in possession of the claimed invention, i.e., complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics. *Enzo Biochem, [Inc. v. Gen-Probe, Inc.*, 323

F.3d 956, 964, 63 USPQ2d 1609, 1613 (Fed. Cir. 2002)].

Here, the analog of claim 1(2) is defined by a complete or partial structure and other physical and/or chemical properties. The examiner concedes that binding is a physical property. There is effectively a partial structure because the DNA encoding it must be capable of hybridizing to the cDNA encoding SEQ ID NO:2 under moderately stringent conditions. Thus, this combination of partial structure and physical and/or chemical properties is sufficient to show that applicant was in possession of the claimed invention.

Binding alone is sufficient to establish the function of serving in affinity chromatography to isolate FAS-IC protein. Note that the affinity chromatography function is mentioned, for example, at page 21, lines 5-9, where it states that affinity chromatography may be used to characterize additional proteins, factors, receptors, etc., which are capable of binding to the MORT-1 protein of the invention. This, of course, includes FAS-IC, as is stated in the following two lines. Accordingly, the present claims fully satisfy the written description guidelines, as it is perfectly acceptable to show that one is in possession of a compound by identifying characteristics that include physical properties.

As to the question of whether or not the full scope of presently-claimed analogs is supported by a sufficient written

description in view of the fact that only a single species is disclosed in the specification, reference is made to the Revised Interim Written Description Guidelines Training Materials (a copy that has recently been downloaded from the PTO website being attached hereto in the Evidence Appendix), Example 14: "Product-by-Function," beginning on numbered page 53 of the attached copy. In that example, the specification exemplified a protein isolated from liver that catalyzed the reaction of A→B, which isolated protein was sequenced and was determined to have the sequence as set forth in SEQ ID NO:3. The specification also contemplated, but did not exemplify, variants of the protein wherein the variant can have any or all of the following: substitutions, deletions, insertions, and additions. The specification indicated that procedures for making proteins with substitutions, deletions, insertions, and additions is routine in the art and provided an assay for detecting the catalytic activity of the protein.

This description in the specification is very similar to the description that appears in the present specification. The present specification exemplifies a MORT protein that binds FAS-IC. The sequence of this protein is specified. The specification contemplates, but does not exemplify, analogs of the protein wherein the variant is encoded by a DNA molecule that hybridizes to the MORT-1 encoding DNA molecule under moderately stringent hybridization conditions. The present specification also

indicates that procedures for making such analogs, including by modification of the DNA sequences encoding them, are routine in the art (see, for example, page 16, lines 5-9) and provides an assay for determining whether any given protein binds to FAS-IC. See, for example, page 34, lines 3-13.

In Example 14 of the Training Materials, the claim is directed to:

> A protein having SEQ ID NO:3 and variants thereof that are at least 95% identical to SEQ ID NO:3 and catalyze the reaction of A→B.

The analysis in the Training Materials acknowledges that procedures for making variants of SEQ ID NO:3 are conventional in the art and that an assay is described which will identify other proteins having the claimed functionality. Moreover, procedures for making variants of SEQ ID NO:3 which have 95% identity to SEQ ID NO:3 and retain its activity were conceded as being conventional in the art. It would, of course, be understood that procedures for making analogs (variants) of the polypeptide of paragraph (1) of claim 1, which analogs are as defined in paragraph (2) of claim 1, are also conventional in the art.

The analysis goes on to point out that all variants of the claim must possess the specified catalytic activity and must have at least 95% identity to the SEQ ID NO:3. Furthermore, because of the "having" language, the protein claimed may be larger than SEQ ID NO:3 or its variant with 95% identity to SEQ ID

NO:3. The analysis points out that the specification contains a reduction to practice of the single disclosed species. The analysis concludes at pages 54-55:

> The specification indicates that the genus of proteins that must be variants of SEQ ID NO:3 does not have substantial variation since all the variants must possess the specified catalytic activity and must have at least 95% identity to the reference sequence, SEQ ID NO:3. The single species disclosed is representative of the genus because all members have at least 95% structural identity with the reference compound and because of the presence of an assay which applicant provided for identifying all of the at least 95% identical variants of SEQ ID NO:3 which are capable of the specified catalytic activity. One of skill in the art would conclude that applicant was in possession of the necessary common attributes possessed by the members of the genus.
>
> Conclusion: The disclosure meets the requirements of 35 USC §112, first paragraph, as providing adequate written description for the claimed invention.

Thus, it is apparent that if the single species disclosed is representative of the genus and an assay is present for identifying the members of the variants that are capable of the specified functionality, the written description requirement is met. Here, the requirement of moderately stringent hybridization conditions and the disclosure of the assay also are sufficient to identify all of the analogs that are capable of the specified binding activity. As in the example, one of skill in the art would conclude applicants were in possession of the

necessary common structure possessed by the members of the genus despite disclaimers of only a single species.

It should further be noted that it is not uncommon to claim analogs by such hybridization language. In this regard, reference is made to Example 9, "Hybridization," in the same Training Materials, beginning at numbered page 35. In that example, the following claim was analyzed for compliance with the written description requirement of 35 U.S.C. §112:

> An isolated nucleic acid that specifically hybridizes under highly stringent conditions to the complement of the sequence set forth in SEQ ID NO:1, wherein said nucleic acid encodes a protein that binds to a dopamine receptor and stimulates adenylate cyclase activity.

The result of this analysis was that the claimed invention is adequately described. As the polypeptide encoded by any such nucleic acid hybridizing under the specified conditions can readily be tested without undue experimentation for ability to block cell proliferation, the experimentation needed to practice the invention was found not undue or unreasonable.

As the Training Materials acknowledge that hybridization language can be acceptable and that a genus of analogs can be claimed based on the disclosure of only a single member of that genus, the situation in this case would warrant a similar analysis, whereby the analogs defined in paragraph (2) of claim 1

are considered to be supported by the written description of the present specification.

The examiner points to the disclosure at page 6, lines 4-5 of the present specification that a certain fragment of MORT-1 does not bind to the MORT-1 protein of SEQ ID NO:2, but does bind to FAS-IC. The examiner states that this could not be used for making an affinity column to isolate MORT-1 protein. If, by inadvertent error in a previous paper in the prosecution of this application, applicants referred to an affinity column to isolate "MORT-1 protein," this error is regretted. It is clear, however, that the claimed function is binding to FAS-IC, and the reference to an affinity column at page 21, lines 5-9, of the present specification can equally apply to the isolation of FAS-IC as an additional protein or receptor that binds to MORT-1 protein. Furthermore, the fact that there may be fractions of SEQ ID NO:2 that do not bind to FAS-IC is irrelevant as the claim requires binding to FAS-IC, and the assay described in the present specification establishes that only those sequences that do bind to FAS-IC are within the scope of the claim.

The examiner states that binding to FAS-IC alone is not a definitive function. However, this is not understood. It is an important function as it allows FAS-IC to be isolated by affinity chromatography. It is definitive as it is easy to determine whether any given analog of MORT-1 has such binding capability.

The examiner states that there is no correlation provided between the function of binding to FAS-IC and the structure of proteins encoded by DNA sequence having the ability to hybridize to the cDNA encoding SEQ ID NO:2 under moderately stringent conditions. However, such a correlation is not necessary in view of the fact that it would not take undue experimentation to subject the proteins that are encoded by the DNA that binds under moderately stringent conditions to a simple binding assay to determine which of those bind to FAS-IC.

As indicated above, those of ordinary skill in the art understand that to bind under moderately stringent conditions, such a DNA would have to have at least about 75% homology. This is a sufficiently defined structure.

For all of these reasons, reversal of the examiner and withdrawal of the rejection under 35 U.S.C. §112, first paragraph, written description, are respectfully urged. It is further urged that the fact that this rejection was withdrawn in the official action of March 4, 2004, and was not re-inserted in the official action of February 25, 2005, and only reinstated on June 6, 2005, diminishes the gravitas of the examiner's argument and the relative arguments should be weighed accordingly.

Determining Which Analogs Bind FAS-IC Would Not Entail Undue Experimentation

The examiner states that applicants have not taught how to make variants that are capable of hybridizing to the cDNA encoding SEQ ID NO:2 under moderately stringent conditions, such that those variants would bind with the intracellular domain of the FAS ligand receptor (FAS-IC). The examiner states that one would not know how to make the claimed variants in view of a lack of adequate teaching in the specification, and in view of the unpredictability of protein chemistry and the DNA sequences that encode proteins.

The examiner's argument fails in view of the fact that it is not necessary to know in advance which variants of the cDNA encoding SEQ ID NO:2 would bind with FAS-IC. It is not necessary to decide whether protein chemistry is predictable or unpredictable. The point is that mutations in the cDNA can be randomly made with all of the random mutations being tested *en masse* for hybridization under moderately stringent conditions. The same is true for fragments of SEQ ID NO:2. All those that hybridize can then be cloned so as to produce an expression product of the DNA in question, and that expression product tested by a very simple binding assay to determine if it binds to the intracellular domain of FAS (FAS-IC). Whatever is found to hybridize under moderately stringent conditions and to encode a polypeptide that binds to

the FAS-IC falls within the scope of the claim. Anything else does not. None of these steps involve undue experimentation.

The specification at page 16 cites Sambrook et al (1989) for standard procedures to prepare analogs. Among such standard procedures are treatment of double-stranded DNA with chemical mutagens, treatment of single-stranded DNA with sodium bisulfite, and treatment of single-stranded DNA with chemicals that damage all four bases, which appear at pages 15.105-15.107 of the Sambrook reference. These are all procedures that would have been well known to those of ordinary skill in the art at the time of the effective filing date of the present application. Simple *in vitro* binding assays are described, for example, at page 34, lines 3-13, of the specification.

The amount of experimentation that may be permitted in order to satisfy the enablement requirement of 35 U.S.C. §112 is discussed in *In re Wands*, 858 F.2d 731, 8 USPQ2d 1400 (Fed. Cir. 1988). In this regard, *Wands* states, 858 F.2d at 736-737, 8 USPQ2d at 1404:

> Enablement is not precluded by the necessity for some experimentation such as routine screening. However, experimentation needed to practice the invention must not be undue experimentation. "The key word is 'undue,' not 'experimentation.'"
>
> The determination of what constitutes undue experimentation in a given case requires the application of a standard

> of reasonableness, having due regard for the nature of the invention and the state of the art. Ansul Co. v. Uniroyal, Inc. [448 F.2d 872, 878-879; 169 USPQ 759, 762-763 (2d Cir. 1971), *cert. denied,* 404 U.S. 1018, 30 L. Ed. 2d 666, 92 S. Ct 680 (1972)]. The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed***.
>
> [Footnotes omitted - the latter quote being from *In re Jackson,* 217 USPQ 804, 807 (Bd. App. 1982)]

Wands goes on to state, 858 F.2d at 737, 8 USPQ2d at 1404:

> Factors to be considered in determining whether a disclosure would require undue experimentation have been summarized by the board in *Ex parte Forman* [230 USPQ 546, 547 (Bd. Pat. App. & Int. 1986)]. They include (1) the quantity of experimentation necessary, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims. [Footnotes omitted]

In analyzing these factors in this case, the conclusion must be reached that the experimentation is not undue. As to the first factor, the quantity of experimentation may be significant, as random mutations would have to be generated, hybridization experiments conducted, and screening conducted of those that are found to hybridize under

moderately stringent conditions using a simple binding assay. However, in the *Wands* case, it was found that routine screening does not necessarily amount to undue experimentation.

With respect to the second factor, the amount of guidance or direction presented, the specification refers to the Sambrook reference, which is the laboratory manual used by everyone of ordinary skill in this art. Everything in it is known and within the skill of those of ordinary skill in the art. Less guidance is needed for well-known techniques. Substantial guidance as to a specific binding screen is provided at page 34.

As to the third factor, the presence or absence of working examples, the binding assay at page 34 is sufficiently detailed to serve as a working example.

As to the fourth factor, the nature of the invention, the nature of the invention is such that substantial experimentation is acceptable. As will be discussed in the following factors, the field of this invention requires a very high level of skill in the art, and practitioners are well inured to screening that takes substantial experimentation quantitatively.

As to the fifth factor, the state of the prior art, moderate stringency hybridization, random mutagenesis and

binding assays are all well-documented in the prior art. The examiner has not doubted this fact, and so it has not been necessary to submit evidence proving it. The present invention does not involve any of these specific techniques *per se*. Their use on the novel DNA sequence of the present invention is the advance made by the present inventors.

As to the sixth factor, the relative skill of those in the art, those of ordinary skill in the art of recombinant DNA technology is very high, usually requiring a Ph.D. and/or substantial laboratory experience. For such persons, a greater amount of experimentation would be considered to be routine than for technologies requiring a lower level of skill in the art.

As to the seventh factor, the predictability of the art, predictability is not relevant here, as no predictability is necessary. One need only do the experiments and screen; the results will provide all of the answers. It is not necessary to predict the answers in advance.

As to the eighth factor, the breadth of the claims, paragraph 2 of claim 1 is not so broad so as to require undue experimentation to find what would fall within it for the reasons as discussed above with respect to all of the other factors.

Accordingly, as in *In re Wands*, analysis of the facts of the present case, considering the factors enumerated in *Ex parte Forman*, leads to the conclusion that undue experimentation would not be required to practice the invention. There was a high level of skill in the art at the time when the application was filed and all of the methods needed to practice the invention were well known.

The examiner states that applicants have not taught how to make variants that are capable of hybridizing to the cDNA encoding SEQ ID NO:2 under moderately stringent conditions such that those variants would have the properties and function of the claimed polynucleotides encoding SEQ ID NO:2. However, this is not required by the present claims. The present claims only require that the variants bind to the intracellular domain of the FAS ligand receptor. The examiner states that not everything that binds to the FAS-IC receptor will cause the receptor to be activated. However, nothing in the claim requires activation of the FAS-IC receptor or any other activity *in vivo*. The claim only requires that the polypeptide encoded by the DNA that binds to SEQ ID NO:2 under moderately stringent conditions bind to FAS-IC. The specification discloses that binding to FAS-IC has a separate *in vitro* utility in affinity chromatography. A protein that binds to FAS-IC can be used to isolate FAS-IC or the FAS receptor by affinity chromatography, and this is a valid utility. A claim

needs only be supported by a single utility. The examiner's comments about whether or not the protein can be used to activate the FAS receptor or otherwise have all of the other properties of MORT-1 are irrelevant. The name of the game is the claim. The claim does not require such activation.

It is noted that the examiner has not rebutted the *Wands* analysis made in the previous filing of the Brief and repeated herein. Further, the examiner has conceded that one could screen for fragments of SEQ ID NO:2 that bind to FAS-IC (see the first sentence of the last paragraph on page 9 of the official action of June 6, 2005).

It should again be noted that following the filing of a Appeal Brief in this case on December 6, 2004, the examiner issued an Official action on February 25, 2005, in which the enablement rejection was not repeated. Accordingly, the examiner was at one point convinced by applicants' arguments. The fact that the examiner in a fifth Official action on the merits should reinstate a rejection despite arguments that were previously found convincing should affect the weight that the Board gives to the examiner's arguments in a negative fashion.

For all of these reasons, reversal of the examiner and withdrawal of the rejection under 35 U.S.C. §112, first paragraph, enablement, are respectfully urged.

CONCLUSION

The claims as submitted are believed to truly set forth the inventive concept of the present invention and to fully comply with the written description and enablement requirements of the first paragraph of 35 U.S.C. §112 and the definiteness requirement of the second paragraph of 35 U.S.C. §112. Accordingly, reversal of the examiner and allowance of claims 1-7, 11 and 14 are earnestly solicited.

Respectfully submitted,

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CLAIMS APPENDIX

This listing of claims includes all of the claims involved in the appeal.

Listing of Claims:

1. An isolated DNA molecule comprising:

(1) a DNA sequence which encodes the MORT-1 protein, having the amino acid sequence of SEQ ID NO:2;

(2) a DNA sequence which encodes an analog of said MORT-1 protein, which analog binds with the intracellular domain of the FAS ligand receptor (FAS-IC), which DNA sequence is capable of hybridization to the cDNA encoding SEQ ID NO:2 under moderately stringent conditions; or

(3) a DNA coding sequence consisting of a DNA sequence which encodes a fragment of said MORT-1 protein which binds with FAS-IC.

2. An isolated DNA molecule in accordance with claim 1, comprising a DNA sequence encoding an analog of said MORT-1 protein which binds with FAS-IC, which DNA sequence is capable of hybridization to the cDNA encoding SEQ ID NO:2 under moderately stringent conditions.

3. A vector comprising a DNA sequence according to claim 1.

4. A vector according to claim 3 which is capable of being expressed in a eukaryotic host cell.

5. A vector according to claim 3 which is capable of being expressed in a prokaryotic host cell.

6. Isolated transformed eukaryotic or prokaryotic host cells containing a vector according to claim 3.

7. A method for producing a polypeptide which binds to the intracellular domain of the FAS-R, comprising growing the isolated transformed host cells according to claim 6 under conditions suitable for the expression of an expression product from said cells, effecting post-translational modifications of said expression product as necessary for obtention of said polypeptide, and isolating said expressed polypeptide.

8-10 (Cancelled).

11. A recombinant animal virus vector encoding a virus surface protein capable of binding a specific target cell surface receptor and further including the sequence of a DNA molecule of claim 1.

12-13 (Cancelled).

14. An isolated DNA molecule in accordance with claim 1 wherein the entire said DNA sequence is a coding sequence encoding said polypeptide.

EVIDENCE APPENDIX

U.S. Patent 5,026,636: Amendment of February 24, 2003, for example, at page 7; such amendment was entered and considered in the next official action on July 3, 2003.

Wood et al, Proc Natl Acad Sci USA 82:1585-1588 (1985): Amendment of February 24, 2003, for example, at page 7; such amendment was entered and considered in the next official action on July 3, 2003.

Hames and Higgins (eds.), Nucleic Acid Hybridisation: A Practical Approach, Chapter 4, IRL Press, Washington (1985): Amendment of February 24, 2003, for example, at pages 7-8; such amendment was entered and considered in the next official action on July 3, 2003.

U.S. Patent 4,968,607: Amendment of February 24, 2003, for example, at page 8; such amendment was entered and considered in the next official action on July 3, 2003.

U.S. Patent 5,171,675: Amendment of February 24, 2003, for example, at page 8; such amendment was entered and considered in the next official action on July 3, 2003.

U.S. Patent 5,198,342: Amendment of February 24, 2003, for example, at page 8; such amendment was entered and considered in the next official action on July 3, 2003.

U.S. Patent 5,262,522: Amendment of February 24, 2003, for example, at page 8; such amendment was entered and considered in the next official action on July 3, 2003.

U.S. Patent 5,237,051: Amendment of February 24, 2003, for example, at page 8; such amendment was entered and considered in the next official action on July 3, 2003.

Ausubel et al (eds.) Current Protocols in Molecular Biology, eds. John Wiley & Sons, Inc., (1987-1998) on page 2.10.11 (Supplement 26, 1994) (including a copy of page 2.6.1 that shows that Supplement 26 is dated 1994): Amendment of February 24, 2003, for example, at page 9; such amendment was entered and considered in the next official action on July 3, 2003.

Revised Interim Written Description Guidelines Training Materials: This is being provided as a citation of authority that may not be readily available to the Board members.

RELATED PROCEEDINGS APPENDIX

None

Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 1585–1588, March 1985
Biochemistry

Base composition-independent hybridization in tetramethylammonium chloride: A method for oligonucleotide screening of highly complex gene libraries

(DNA melting)

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Communicated by Gary Felsenfeld, October 31, 1984

ABSTRACT **An oligonucleotide hybridization procedure has been developed that eliminates the preferential melting of A·T versus G·C base pairs, allowing the stringency of the hybridization to be controlled as a function of probe length only. This technique, which uses tetramethylammonium chloride, is especially helpful whenever a highly complex library is screened with a pool of oligonucleotide probes, which usually vary widely in base composition. The procedure can also be applied advantageously whenever an exact match to an oligonucleotide probe is desired, such as in screening for clones having as little as a single-base alteration generated by *in vitro* mutagenesis.**

Short oligonucleotide probes [range, 14–20 base pairs (bp)] are commonly used to screen libraries of cloned DNA for genes of interest (1–3). Typically, these probes are pools representing all possible codon choices for a short amino acid sequence. Although this method has been successful, there is considerable uncertainty in the hybridization conditions because the binding of the oligonucleotides depends on two factors: (*i*) the length of the hybrid formed and (*ii*) the G·C content of the probe. Empirically determined formulas allow for estimation of the oligonucleotide dissociation temperature (T_d) (4); however, these methods can be unsatisfactory when screening with pools of oligonucleotides. Although the length of the probes in the pool is constant, the individual probes differ considerably in G·C content, making suitably stringent and selective hybridization conditions difficult to find for all members of the pool. Thus, a large number of false positives can occur when screening highly complex libraries for genes of low abundance.

We describe here the use of tetramethylammonium chloride (Me_4NCl) in the hybridization of oligonucleotide probes to eliminate the dependence of T_d on the G·C content of the probe, reducing the problem to a simple dependence on length of the hybrid. Tetraalkylammonium salts were found some years ago to bind to A+T-rich polymers of DNA (5) and have been used to abolish the preferential melting of A·T versus G·C base pairs for fragments of DNA (6). Me_4NCl binds selectively to A·T base pairs, thus displacing the dissociation equilibrium and raising the melting temperature. At 3.0 M Me_4NCl, this displacement is sufficient to shift the melting temperature of A·T base pairs to that of G·C base pairs (6). For the melting of long DNA, this shift results in a remarkable sharpening of the melting profile. Natural DNAs that melt over a range of 5 to 10°C in the presence of Na^+ melt within 1°C in Me_4NCl (6–8). The data presented here show the utility of hybridizations with Me_4NCl when oligonucleotide probes are used. The T_d values of probes from 11 to >1000 bp have been determined so that hybridization conditions for probes of various lengths can be chosen easily. This method is applicable to a variety of circumstances in which an exact match to a probe is desired.

METHODS

DNA Synthesis, Binding, and Labeling. In this procedure, nitrocellulose filters are used routinely with DNA spotted directly or bound (9) from bacteriophage λ or M13 plaques or from plasmid-containing bacterial colonies. Filters removed from bacteriophage or bacterial colony plates are treated with 0.5 M NaOH/1.5 M NaCl, which is neutralized with 1 M Tris·HCl, pH 7.5/1.5 M NaCl. DNA spots are made by denaturing the sample with 0.3 M NaOH, neutralizing with 0.6 M Tris·HCl, pH 7.4/1.5 M NaCl, and immediately spotting on nitrocellulose filters previously soaked in 3.0 M NaCl/0.3 M Na citrate (20× NaCl/Cit) and dried. The DNA-containing filters are baked for 2 hr at 60–80°C in a vacuum oven. Oligonucleotide probe pools were synthesized from trimers by the triester method (10). Pools 9.3 and 9.4 were end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (9).

Hybridization and Me_4NCl Wash Procedure. In this procedure, an initial nonstringent hybridization with radiolabeled probe is followed by washing with 3.0 M Me_4NCl to control the stringency of the hybridization. The filters are prehybridized in 6× NaCl/Cit/50 mM sodium phosphate, pH 6.8/5× Denhardt's solution (1× Denhardt's solution is albumin/polyvinylpyrrolidone/Ficoll, each at 0.2 mg/ml) containing boiled sonicated salmon sperm DNA at 0.1 mg/ml for 4–16 hr at 37°C. The filters are hybridized overnight at 37°C in the same solution plus dextran sulfate at 100 mg/ml and the pool of end-labeled oligonucleotide probes (each at 180 pM; 560,000–1,680,000 cpm/pmol) (11). [For a 17-mer probe this is 1 μg/liter (100–300 cpm/pg). For a pool of 16 probes, the probe concentration is 16 μg/liter.] The filters are rinsed three times with 6× NaCl/Cit at 4°C and washed twice for 30 min with 6× NaCl/Cit at 4°C. The filters are then rinsed with the Me_4NCl wash solution at 37°C to remove the NaCl/Cit [the NaCl/Cit must be substantially removed because Na^+ will compete for Me_4N^+ binding (12)] and washed twice for 20 min with the Me_4NCl wash solution at the desired temperature. In screening experiments, we typically use a temperature 2–4°C below the T_d shown in Fig. 3. The wash temperature needs to be well controlled (±1°C); suitable heat exchange can be obtained only in a shaking or circulating water bath. The Me_4NCl wash solution is 3.0 M

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Abbreviations: bp, base pair(s); T_d, dissociation temperature; Me_4N^+, tetramethylammonium ion; Et_4N^+, tetraethylammonium ion; NaCl/Cit, 0.15 M NaCl/0.015 M Na citrate.

Me_4NCl/50 mM Tris·HCl, pH 8.0/2 mM EDTA containing NaDodSO$_4$ at 1 mg/ml. Me_4NCl is purchased from Aldrich and made up as a nominal 5 M stock solution. Since Me_4NCl is hydroscopic, the actual molar concentration (c) is determined from the refractive index (η) by the formula $c = (\eta - 1.331)/0.018$ (12). The concentration needs to be fairly precise since the T_d varies with the Me_4NCl concentration (6).

The prehybridization and hybridization described above are used for oligomers in the range 14–50 nucleotides. For shorter oligonucleotides, a room temperature hybridization is preferable. For oligonucleotides of ≥ 50 nucleotides, the prehybridization and hybridization are as above except that formamide at 400 ml/liter is included at 37°C. In the range 46–75 bp, we have compared hybridization with and without formamide; the two give equal results. Nitrocellulose filters become fragile in washes at 85°C and above. If frequent use is made of high-temperature washes, it is possible to use 2.4 M tetraethylammonium chloride (Et_4NCl) rather than 3.0 M Me_4NCl. Et_4NCl has the same desirable effect on DNA melting as Me_4NCl [at least with long DNA (6, 7)], but the entire melting profile is shifted 33°C lower. Although with Et_4NCl the melting temperature of short oligonucleotides is inconvenient (below room temperature), it could be useful for 50- to 200-bp probes.

RESULTS AND DISCUSSION

The results of hybridization and melting of four oligonucleotide probes in the commonly used 6× NaCl/Cit system (1, 2, 11, 13) are compared with those in 3.0 M Me_4NCl in Fig. 1. These probes are 17-mers, and their hybridization is determined by the stretch of contiguous match, the hybridizing length, rather than by the percentage homology because even a single internal mismatch in probes of this length lowers the T_d by 5–10°C (refs. 11 and 13; unpublished observations). A series of DNA spots was hybridized under nonstringent conditions and washed at temperature intervals of 2 or 3°C. As shown in Fig. 1*A*, the melting in 6× NaCl/Cit does not depend linearly on the hybridizing length. In particular, the probe with the 13-bp hybridizing region melts some 5°C higher than the 15-bp probe and at about the same temperature as the 16-bp probe. This is because the probe with the 13-bp hybridizing region is 69% G-C while the 15- and 16-bp probes are 40 and 44% G-C. These melting results in 6× NaCl/Cit contrast with those in 3.0 M Me_4NCl (Fig. 1*B*) in which the probes melt according to their hybridizing length. These results illustrate that the preferential melting of A·T base pairs is abolished in oligonucleotide hybridizations in 3.0 M Me_4NCl, as previously shown for long DNA (6). The T_d values derived from the data in Fig. 1 are plotted as a function of hybridizing length in Fig. 2.

To extend the utility of the method over a wide range of probe lengths, the T_d values in 3.0 M Me_4NCl were determined as a function of length for 18- to 1374-bp restriction fragments of pBR322. These fragments were end-labeled, hybridized to a series of pBR322 DNA dots, and washed at various temperatures with 3.0 M Me_4NCl. The T_d, length, and G-C content of these fragments are shown in Table 1. When plotted as a function of length, the T_d values lie on a smooth curve from 45 to 93°C in spite of the widely varying G-C content (31–66%) of the probes (Fig. 3). This further demonstrates the lack of dependence of the T_d on the G-C content. The T_d is essentially independent of length above 200 bp, and the limiting T_d of 93°C agrees with that reported for long DNA (6, 7). From either Fig. 3 or Table 1 one can determine the hybridization wash temperature so that the stringency can be based solely on the probe length.

The utility of hybridization in Me_4NCl is perhaps best illustrated by the following example based on our own experience: A pool of sixteen 17-mers representing all possible codons for the protein sequence Glu-Cys-Trp-Cys-Gln-

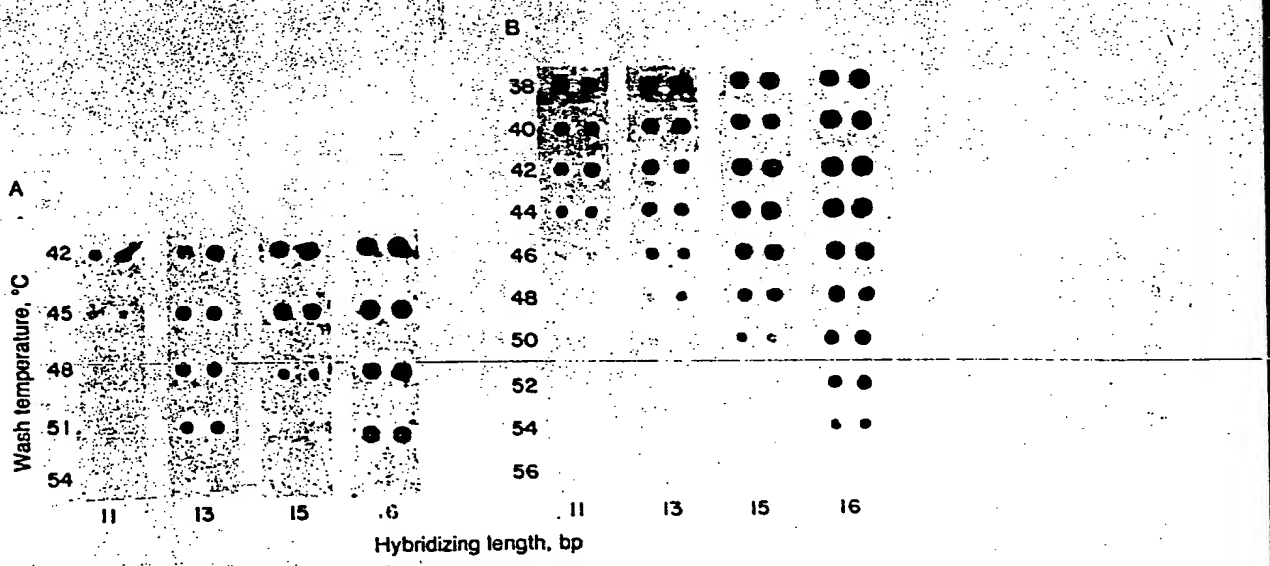

FIG. 1. Hybridization of oligonucleotides of various hybridizing lengths washed in 6× NaCl/Cit or 3.0 M Me_4NCl at increasing temperatures. Spots containing 125–250 ng of bacteriophage λ20a or λ21a DNA were made on nitrocellulose membranes, and these DNA spots were hybridized to ^{32}P-labeled probe pools 9.3 or 9.4. Pool 9.3 contains sixteen 17-mer probes of the sequence 5′ G-C-$\frac{C}{T}$-T-G-$\frac{A}{G}$-C-A-C-C-A-$\frac{A}{G}$-C-A-$\frac{C}{T}$-T-C. Pool 9.4 contains sixteen 17-mers with the sequence 5′ T-A-$\frac{C}{T}$-T-G-$\frac{C}{T}$-T-T-C-C-A-$\frac{A}{G}$-A-A-$\frac{C}{T}$-T-C. λ20a and λ21a are clones isolated from a bovine genomic library (14) by conventional hybridization with 6× NaCl/Cit washes to pools 9.3 and 9.4 (unpublished data). Pool 9.3 has a contiguous match of 11 bp to λ21a, A-*C-T-T-G-A-C-A-C-C-A-A-A-C-T*-C-A (the nucleotides in italic type are those of the λ21a sequence that match the probe), and a contiguous match of 13 bp to λ20a, *G-C-T-T-G-G-C-A-C-C-A-G-C-T-T*-G-C. Pool 9.4 has a 15-bp match to λ21a, *T-G-T-T-G-C-T-T-C-C-A-G-A-A-T-T-C*, and a 16-bp match to λ20a, A-*A-C-T-G-C-T-T-C-C-A-A-A-A-C-T-C*. Duplicate hybridized spots were washed at the indicated temperatures in 6× NaCl/Cit containing NaDodSO$_4$ at 1 g/liter twice for 10 min (*A*) or in 3.0 M Me_4NCl (*B*) as described in *Methods*. Spots are shown after autoradiography overnight at −70°C with DuPont Lightning Plus intensifying screens.

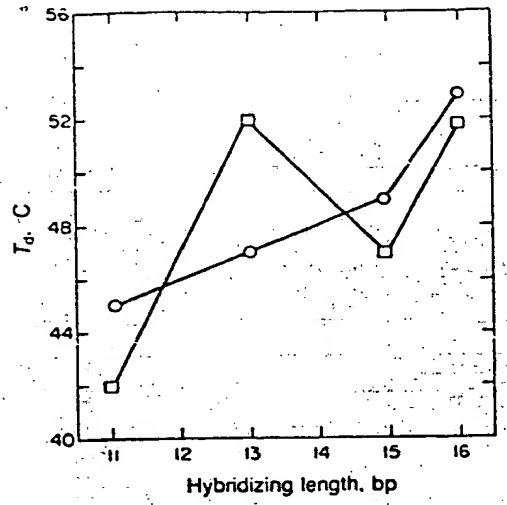

FIG. 2. T_d of oligonucleotide probes as a function of hybridizing length in 6× NaCl/Cit (□) or 3.0 M Me_4NCl (○). T_d values were determined visually from the data in Fig. 1 as the temperature at which half the intensity remained and plotted as a function of hybridizing length. T_d values are used rather than melting temperature (T_m) values, which are determined under conditions of thermodynamic equilibrium.

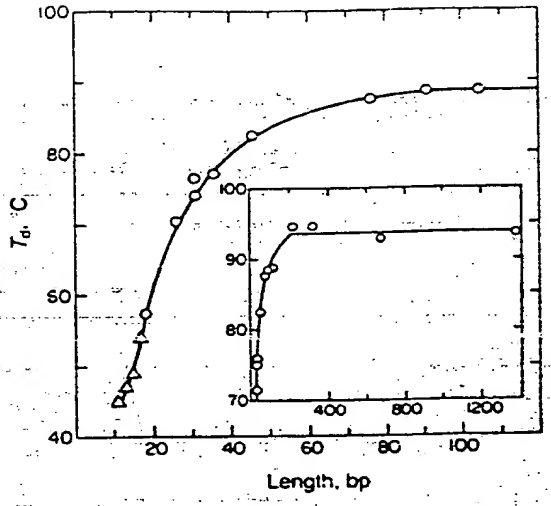

FIG. 3. T_d in 3.0 M Me_4NCl as a function of probe length. A series of pBR322 DNA spots was made on nitrocellulose membranes, and these were hybridized and washed with 3.0 M Me_4NCl. Washes of the duplicate spots were carried out at 3°C intervals. Hybridization probes of various lengths were made by cutting pBR322 DNA with *Sau*3AI, treating with alkaline phosphase, labeling with T4 polynucleotide kinase and [γ-^{32}P]ATP, and isolating the labeled fragments from a polyacrylamide gel (9). These double-strand probes were boiled before hybridization. From an autoradiogram of the hybridized spots (analogous to that shown in Fig. 1), a T_d was determined and plotted for each fragment (○). Also shown are melting data in Me_4NCl from Fig. 2 (△).

Ala was synthesized (probe 9.3; see Fig. 1). This pool consists of oligonucleotides ranging in G-C content from 47 to 71%. In 6× NaCl/Cit, a temperature that allows hybridization of the probe having the lowest G-C content will also allow hybridization of regions as short as 12 or 13 bp in the probes having the highest G-C contents. When screening a highly complex library, such as those derived from mammalian genomes, this can lead to a large number of false positives. The expected number of hybridizing sequences per haploid genome at random, N, is approximately

$$N = C(2)(n - h + 1)(p)/4^h,$$

where C = complexity of the genome in bp, n = probe length, h = contiguous hybridization match, and p = pool size (see *Appendix* for derivation). Thus, for a pool of sixteen 17-mers where matches as short as 13 are allowed ($C = 3.3 \times 10^9$, $n = 17$, $h = 13$, $p = 16$), the expected number of clones at random is 8000. These clones represent a large number of false positives. However, with Me_4NCl, a hybridization temperature can be selected in which only 17- and perhaps some 16-bp matches are allowed (52–54°C). In this case, the expected number of random clones is only 6 (or 49 for 16-bp matches), a number small enough to be searched through for the correct clone by DNA sequence analysis or by a precise melting in Me_4NCl as described below. As noted in the *Appendix*, the application of the formula for N to hybridization in 6× NaCl/Cit is not quite correct because all matches of length 13 (not just those of high G-C content) are counted. Thus, perhaps only 1000–4000 positives would be found with 6× NaCl/Cit hybridization. In any event, the number of false positives due to probes of high G-C content can be reduced significantly by using Me_4NCl.

Table 1. Probe G-C content and T_d in 3.0 M Me_4NCl

Hybridizing length, bp	G-C content, %	T_d in 3.0 Me_4NCl, °C
11	45	44–45
13	69	47
15	40	49–50
16	44	53–54
18	44	57–58
27	63	70–71
31	58	74 (76)
36	53	77
46	54	82–83
75/78	48/42	87–88
91	66	88–89
105	31	88–89
207	55	94–95
317	65	94–95
665	46	93
1374	55	93–94

Probes having hybridizing lengths of 11–16 bp are described in the legend to Fig. 1. Those 18–1374 bp long are *Sau*3AI fragments of pBR322.

In addition to screening highly complex libraries, the Me_4NCl procedure may be adapted advantageously in a variety of other situations. For example, it can be used to select the best candidates for DNA sequencing. If a number of candidate clones have been obtained, those that match the probe most closely can be found by plating out replicas of the clone or spotting the DNA, hybridizing to an oligonucleotide probe, and washing with 3.0 M Me_4NCl at increasing temperature in the manner of Fig. 1. We have used six duplicate spots of the DNA and washed at 2°C intervals to find the clones that match the probe exactly, thus eliminating unnecessary DNA sequencing. In this way, the match with the probe can be determined to ±1 bp in the 14- to 20-nucleotide range. This technique can be especially precise if standards of known hybridizing length are included at the same time. Another application of this method has been in screening of M13 clones that had been mutagenized *in vitro* (15). In this procedure, a 20-mer having one or more centrally located base changes is synthesized and used to generate M13 plaques that are screened for an exact match

with the same oligonucleotide as the probe. Since even a single-base-pair internal mismatch decreases the T_d of the hybrid 5 to 10°C (11, 13), the correct mutant can be found by screening in Me_4NCl, using the wash conditions shown in Fig. 3. Furthermore, for different oligonucleotides, empirical determination of a suitable wash temperature is unnecessary. Finally, the method can be used to select one particular gene out of a closely related gene family. For example, a clone for the γ subunit of mouse nerve growth factor was selected from a family of closely related kallikrein-like cDNA clones by hybridization in Me_4NCl (16). In this case, the probe was a pool of thirty-two 14-mers based on protein sequence data.

In summary, hybridization in Me_4NCl is useful whenever an exact match with an oligonucleotide probe is desired. This method is being used routinely for oligonucleotide hybridization to bacteriophage λ or M13 plaques, plasmid-containing bacterial colonies, and DNA spots. While we have not yet used the procedure for genomic blot hybridization with oligonucleotide probes, it should be useful in detecting single-base mismatches in hybridizations to genomic DNA (17). Here again, the hybridization conditions would not need to be determined empirically.

APPENDIX

Derivation of N, the Approximate Number of Sequence Matches Expected per Haploid Genome at Random. Let C = complexity of the genome in bp, n = probe length, h = contiguous hybridizing length, and p = pool size; then, considering the genome as random sequence, $N = C(2)f$, where f is the frequency of some sequence at random. The frequency times the number of bp, C, times 2 (the sequence of interest could be on either strand) is N, the number of sequence matches. The frequency of a particular sequence of length n, where every nucleotide matches, is $1/4^n$. If only h contiguous bp of a probe of length n match, then the frequency of sequence match is $1/4^h$ times the number of contiguous length h matches in the probe. For $n = 17$ and $h = 15$, the number of contiguous matches is $(n - h + 1) = 3$. Thus, for a single probe of length n, where contiguous matches of length h are allowed, the frequency of a sequence match is $(n - h + 1)/4^h$. If a pool of p such probes is used, then $f = p(n - h + 1)/4^h$ and $N = C(2)(n - h + 1)p/4^h$.

The expected number of clones at random, N, given by this formula is about the number we have found in screening a bovine genomic library by using probe pools 9.3 and 9.4 (Fig. 1). We screened 750,000 plaques of a library representing 3.5 haploid genomes with 3.0 M Me_4NCl washes at 50°C and found 80 and 130 positives per haploid genome for the two pools. For these conditions, we would expect 16- and 17-bp matches with perhaps some matches as short as 15 bp. For $C = 3.3 \times 10^9$, $n = 17$, $p = 16$, and $h = 16$ or 15, the formula gives $N = 49$–295 expected random positives, approximately the number found. Application of this formula, based on the contiguous match, is especially appropriate for hybridizations in Me_4NCl where the match length can be controlled. Use of the formula with hybridization in 6× NaCl/Cit is less exact because the number of hybridizing sequences in a pool is affected by the G·C content.

This formula can also be used to estimate the number of random matches to be found in cDNA or other libraries. For this purpose, C can be set to the total number of bp screened (assuming the clones are independent), and the number of hybridizing clones expected at random can be calculated. Clearly, this will overestimate the number of randomly hybridizing clones for most cDNA libraries because some clones are represented many times. However, it can still be used to obtain a rough approximation of what to expect for a particular sized library, oligonucleotide probe, and hybridization stringency.

Note Added in Proof. Recently we have successfully used the Me_4NCl procedure for genomic blot hybridization. The frequency in the population of a single base polymorphism in the factor VIII gene (18) was determined by the hybridization of duplicate lanes of human DNA to two 21-mer probes that differ only at a single central residue. Wash conditions based on Fig. 3 (58°C) revealed a single band with no lane-specific background and clearly showed that 7 of 11 individual DNAs have one sequence while the remaining 4 have the other.

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Nucleic acid hybridisation

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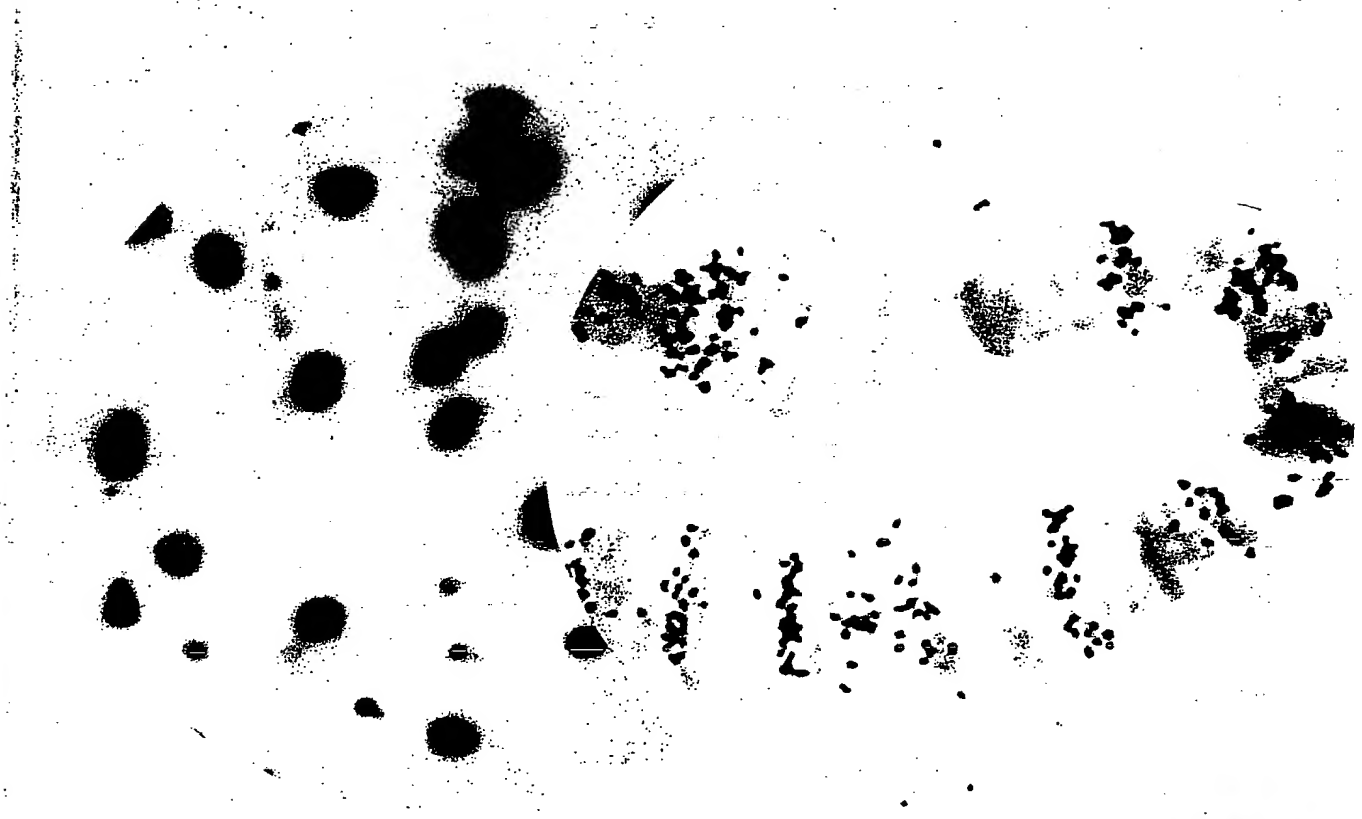

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CHAPTER 4

Quantitative Filter Hybridisation

MARGARET L.M. ANDERSON and BRYAN D. YOUNG

1. INTRODUCTION

An application of nucleic acid hybridisation, which is of central importance to genetic engineering and is finding increasing use in molecular biology, is filter hybridisation. The technique is derived from the classical experiments of Gillespie and Speigelman (1). Denatured DNA or RNA is immobilised on an inert support, for example nitrocellulose, in such a way that self-annealing is prevented, yet bound sequences are available for hybridisation with an added nucleic acid probe. To facilitate analysis, the probe is labelled, often with ^{32}P. Hybridisation is followed by extensive washing of the filter to remove unreacted probe. Detection of hybrids is usually by autoradiography although, when the hybrids are sufficiently radioactive, scintillation counting can be used. The procedure is widely applicable, being used for phage plaque and bacterial colony hybridisation, Southern and Northern blot hybridisation, dot blot hybridisation and hybrid selection (see other chapters in this volume).

While solution hybridisation is the standard method for quantitative measurements of sequence complexity and composition (2), there are practical difficulties when the number of samples is large. By contrast, dot blot hybridisation is ideally suited to the analysis of multiple samples. The technique has the added advantage that it is easy to prepare replicate filters allowing many filter-bound sequences to be analysed at the same time, for example with different probes or under different hybridisation and washing conditions. Dot blot hybridisation can be used qualitatively since it is capable of great discrimination, as exemplified by the ability to distinguish between closely similar members of multigene families (3,4). It can also be used quantitatively with appropriate calibration (5), but it is most commonly used as a semi-quantitative method for determining the relative levels of sequences in different samples. As we shall see, its use is limited by the low rate of hybridisation and by a level of sensitivity which makes it less useful than solution hybridisation for analysing rare sequences.

In this chapter we will first discuss theoretical aspects of filter hybridisation (Sections 2 – 5) and then describe practical aspects (Sections 6 – 14). To make clear the distinction between sequences which are in solution and those which are filter-bound, we will use a different nomenclature from that in the previous chapter. Subscripts 's' and 'f' will be used for nucleic acid in solution and filter-bound nucleic acid, respectively.

2. KINETICS OF FILTER HYBRIDISATION

Nucleic acid hybridisation depends on the random collision of two complementary sequences. As described in the previous chapter, the time course of the reaction in

solution is determined by the concentration of the reacting species and by the second order rate constant, k. The stability of the duplex formed is dependent on its melting temperature, T_m. For hybridisation of perfectly-matched complementary sequences in solution, equations have been derived which describe the process fairly precisely (e.g., Chapter 3, Equations 4, 5 and 10). Detailed investigations have determined the effects of changes in reaction conditions for solution hybridisation, so that values for k and T_m can be calculated with some confidence. In contrast, filter hybridisation has been less extensively studied, and the parameters affecting the rate and extent of reaction are less well understood. Calculations made from solution hybridisation are not necessarily valid for filter hybridisation, although changes in reaction conditions probably have a similar qualitative effect.

The hybridisation of a denatured nucleic acid probe in solution to a filter-bound nucleic acid is a function of two competing reactions, *viz.* the reassociation of sequences in solution and the hybridisation to filter-bound DNA or RNA. (Since the filter-bound nucleic acid is immobilised, reassociation of bound sequences does not occur.) The rate of disappearance of single strands may be expressed by the equation:

$$\frac{-d[C_s]}{dt} = k_1[C_f][C_s] + k_2[C_s]^2 \qquad \text{Equation 1}$$

where C_f is the concentration of filter-bound nucleic acid sequence, C_s is the concentration of nucleic acid probe in solution, k_1 is the rate constant for the hybridisation reaction on the filter and k_2 is the rate constant for the reassociation in solution. The term $k_1[C_f][C_s]$ represents the filter hybridisation while the term $k_2[C_s]^2$ represents reassociation in solution.

A variety of factors affect the rate constants (see Section 3). The rate constant should be the same for both reactions (i.e., $k_1 = k_2$) provided that:

(i) the nucleation rates at the filter and in solution are the same

(ii) the effective molecular weight of the nucleic acid species in solution is smaller than that of the filter-bound nucleic acid, since the rate constant for DNA-DNA reassociation is dependent on the size of the smaller fragment (6,7), at least in solution.

Equation 1 predicts that the initial rate of hybridisation is proportional to the concentrations of both the probe in solution and the filter-bound sequences. When $[C_f]$ is much higher than $[C_s]$, as in dot blots of plasmid DNA (this Chapter, Section 6.2.1 and Chapter 5, Section 3.1) or in hybrid selection (Chapter 5, Section 3.2), the solution reassociation term can be ignored and Equation 1 simplifies to the pseudo-first order reaction:

$$\frac{-d[C_s]}{dt} = k_1[C_f][C_s] \qquad \text{Equation 2}$$

where $[C_f]$ is constant.

On integration, this gives:

$$\frac{[C_s]_t}{[C_s]_0} = e^{-k_1[C_f]t} \qquad \text{Equation 3}$$

where $[C_s]_t$ is the value of $[C_s]$ at time t.

While it has been shown experimentally that the initial hybridisation rate is proportional to $[C_s]$, the relationships in Equations 1 and 2 do not describe exactly the dependency of the hybridisation rate on $[C_f]$. At low values of $[C_f]$, the initial rate of hybridisation is proportional to $[C_f]$, but the rate does not increase linearly at higher values (8—10). This is explained by the fact that filter hybridisation depends on two processes, diffusion of the probe to the filter and hybridisation at the filter. It is thought that at low values of $[C_f]$, the hybridisation reaction itself is the rate-limiting step, whereas at high values of $[C_f]$ hybridisation is so fast that the solution surrounding the filter becomes depleted of probe and the overall reaction is then limited by diffusion of the probe to the filter. Flavell *et al.* (9) have shown that at higher values of $[C_f]$ the rate equation should incorporate a term, J, to take diffusion of the probe into account. Therefore, at high values of $[C_f]$, Equation 1 can be replaced by an equation of the form:

$$\frac{-d[C_s]}{dt} = J + k_2[C_s]^2 \qquad \text{Equation 4}$$

where $k_1\,[C_f]\,[C_s] > J > 0$. The diffusion term J is a function of the diffusion coefficient of the probe and the concentration gradient of the probe. The relationship between J and $k^2[C_s]^2$ determines whether reassociation of the probe is an important factor. When J is $\leq k_2[C_s]^2$, reassociation will be significant.

Since many filter hybridisation experiments aim to hybridise the maximum amount of probe to excess sequences on the filter, the relationship given in Equation 4 is important. The overall hybridisation reaction will be speeded up by factors which increase diffusion of the probe to the filter, for example, using a small probe, high incubation temperature, low reaction volume and shaking the reaction vessel. The rate constant k_1 can be determined by two methods.

(i) From initial reaction rates. Rearranging the terms in Equation 2,

$$k_1 = \frac{v_i}{[C_f][C_s]}$$

where v_i is the initial rate of reaction. So in a filter-bound DNA excess reaction, a plot of the reciprocal of the percentage of added probe which has hybridised to the filter *versus* the reciprocal of the time of reaction will give a straight line with a slope of $1/k_1$ (Chapter 3, Section 3.3.1). This holds true for both double-stranded and single-stranded probes, although a small correction may have to be made in the value of $[C_s]$ for reassociation of the probe in solution.

(ii) From measuring $t_{1/2}$, that is, the time when $[C_s]_t/[C_s]_0$ is 0.5. For a filter-bound DNA-excess reaction (pseudo-first order kinetics) a plot of log [fraction of the

probe remaining single-stranded] against the time of reaction will give a straight line. The $t_{½}$ can be read off the graph and substituted in Equation 3 to give:

$$[C_f]t_{½} = \frac{\ln 2}{k_1} \qquad \text{Equation 5}$$

or

$$k_1 = \frac{0.693}{[C_f]t_{½}} \qquad \text{Equation 6}$$

Experimentally, values for k_1 obtained from $t_{½}$ measurements and from initial rate data for hybridisation of a simple DNA probe to filter-bound DNA are in good agreement. However, the values obtained are 10 times lower than those obtained for solution hybridisation of the same DNAs (9). The reason may be that only a fraction of the DNA bound to the filter is accessible for nucleation, although all the DNA can effectively participate in hybrid formation. Thus the concentration term $[C_f]$ used to calculate the rate constant may be incorrect and k_1 (hybridisation) may actually be equal to k_2 (reassociation). Alternatively, rate constants may be lower for filter hybridisation. As a consequence of binding nucleic acid sequences to the filter, steric restraints may retard the formation of stable nucleation complexes.

Equations 1 and 4 show that one of the factors affecting the kinetics of a filter hybridisation reaction is reassociation of the probe. This variable is often overlooked but its effects can be large and can cause problems in interpreting results. It has been shown that as much as 20—30% of the input DNA probe can be unavailable for hybridisation due to reassociation (9). A second complication is that the probe may form concatenates of partially-reassociated duplexes with single-stranded regions which can hybridise to filter-bound sequences. Again the effects are not negligible. Flavell *et al.* (11) showed that 10% of the added denatured, double-stranded DNA which hybridised to a filter containing single-stranded DNA represented homologous rather than complementary sequences. Similar problems can arise in DNA-RNA hybridisation experiments with self-complementary transcripts. In order to minimise these complications, it is desirable to choose reaction conditions which facilitate diffusion of probe to the filter and favour hybridisation over reassociation, that is, use of a small probe (preferably single-stranded), small reaction volume, a low concentration of probe in solution and a high reaction temperature.

3. FACTORS AFFECTING THE RATE OF FILTER HYBRIDISATION

3.1 Concentration of the Probe

There has been no systematic study of effects of the concentration of probe on the rate of hybridisation at the filter and on the yield of duplex. However, the following points should be noted.

3.1.1 *Double-stranded Probe in Excess*

If the probe is a simple double-stranded sequence, Equation 1 predicts that at high $[C_s]$ values, reassociation of the probe should be favoured over hybridisation to the bound nucleic acid. Therefore, as incubation continues, the reaction will change from being

in probe excess to being in filter-bound excess where, as we have seen, the kinetics are different. Increasing the concentration of probe in solution, $[C_s]$, will increase the initial rate of hybridisation at the filter and the proportion of filter-bound sequences in duplex will increase, but not dramatically. For DNA probes and filter-bound RNA, as in RNA dot blots, high concentrations of formamide can be used to suppress reassociation in solution (see Section 4.1.2).

3.1.2 *Single-stranded Probe*

Whether in excess or not, there is no reassociation of a single-stranded probe in solution unless there are regions of extensive self-complementarity. The rate of hybridisation to the filter and the amount of hybrid formed should increase with increase in $[C_s]$. It is important to note, however, that the probe concentration should not be increased without limit. If more than about 100 ng ^{32}P-labelled probe per ml is used, non-specific irreversible binding to the filter occurs.

3.2 Probe Complexity

For solution hybridisation, the rate of reassociation of DNA is an inverse function of its complexity, so that the more complex the DNA, the slower the rate of reassociation (2,12). Extending this to filter hybridisations, the rate of reassociation of the probe should fall when the complexity of the DNA increases and its effective $[C_s]$ decreases. This is indeed what is observed (9). In contrast, two effects of complexity are seen for hybridisation of the probe to filter-bound nucleic acid sequences. When $[C_f]$ is low, the rate of hybridisation is inversely proportional to complexity over a 400-fold range, indicating that the reaction is controlled by the nucleation step. However, when the hybridisation reaction is limited by diffusion of the probe to the filter, that is when $[C_f]$ is high, the rate of reaction is independent of complexity (9).

3.3 Molecular Weight of the Probe

For DNA-DNA hybridisation in solution, the rate is directly proportional to the square root of the molecular weight of the nucleic acid (12) and this also describes the reassociation of the probe in solution during filter hybridisation (9). However, the effect of the molecular weight of the probe on the rate of hybridisation to filter-bound sequences contrasts sharply with that found in solution. Two situations can occur. When $[C_f]$ is low compared with $[C_s]$, that is, a nucleation-limited reaction, the rate of hybridisation is independent of the molecular weight (9,10). When $[C_f]$ is high compared with $[C_s]$, that is, diffusion-limited filter hybridisation, the rate of hybridisation is inversely proportional to the molecular weight of the probe, but there are insufficient data for an exact relationship to be formulated. The observed rate of hybridisation is significantly depressed by an increase in the molecular weight of a single-stranded probe (which is not capable of reassociation). This effect is even more pronounced when a double-stranded probe is used. This is because the combined effects of a lower rate of hybridisation and the increased rate of reassociation, which accompanies an increase in molecular weight of the sequences in solution, result in lower observed rates of hybridisation and a reduced final yield of hybrid. The difference in dependence on molecular weight of the two types of filter hybridisation is not understood.

3.4 Base Composition

The base composition of nucleic acids affects the rate of hybridisation, the rate increasing with increasing % G+C. However, the effect is small (12) and can be ignored in practice.

3.5 Temperature

The temperature of reaction affects the rate of any hybridisation reaction (13). Typically a bell-shaped temperature dependence curve is obtained. At 0°C, hybridisation proceeds extremely slowly, but as the temperature is raised, the rate increases dramatically to reach a broad maximum which is 20−25°C below T_m for DNA-DNA annealing. At higher temperatures the duplex molecules tend to dissociate so that as the temperature approaches T_m −5°C, the rate is extremely low. The relationship applies to the formation of both well-matched and poorly-matched hybrids although the curve is displaced towards lower temperatures for mismatched duplexes (14). So, ideally, hybridisations should be carried out at a T_i (incubation temperature) that is 20−25°C below T_m. In practice, for well-matched hybrids, the hybridisation reaction is usually carried out at 68°C in aqueous solution and at 42°C for solutions containing 50% formamide. For poorly-matched hybrids, incubation is generally at 35 − 42°C in formamide-containing solutions.

A similar dependence has been shown for RNA-DNA hybridisations (10), but here the maximal rate of hybridisation is obtained at some 10−15°C below the T_m of the hybrids.

3.6 Formamide

Formamide decreases the T_m of nucleic acid hybrids (see Section 4.1). This is a very useful property because by including 30−50% formamide in the hybridisation solution, the incubation temperature, T_i, can be reduced to 30−42°C. This has several practical advantages: the probe is more stable at lower temperatures, there is better retention of non-covalently-bound nucleic acid on the filter and nitrocellulose filters are less likely to disintegrate at the lower temperature.

Concentrations of formamide between 30 and 50% apparently have no effect on the rate of filter hybridisation and 20% formamide reduces the rate by only about one-third (15). On the other hand, a concentration of 80% formamide is thought to depress the rate constant for hybridisation in solution at least by a factor of three for DNA-DNA duplexes and by a factor of 12 for RNA-DNA hybrids (16). Qualitatively similar results are likely to occur in filter hybridisation.

Formamide can be used to alter the stringency of the reaction conditions. By holding T_i constant and varying the concentration of formamide, different effective temperatures are obtained. Effective temperatures as low as 50°C below the T_m of perfectly-matched hybrids can be reached which allows detection of homologies with as much as 35% mismatching (15).

3.7 Ionic Strength

At low ionic strength, nucleic acids hybridise very slowly, but as the ionic strength increases, the reaction rate increases. The effect is most dramatic at low salt concen-

trations (<0.1 M Na^+) where a 2-fold increase in concentration increases the rate 5- to 10-fold. Above 0.1 M Na^+ the rate dependence is less, but still marked up to about 1.5 M Na^+ (12,17).

High salt concentrations stabilise mismatched duplexes, so to detect cross-hybridising species, the salt concentration of hybridisation and washing solutions must be kept fairly high. Washing is therefore generally carried out using 2–6 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0).

3.8 Dextran Sulphate

Wetmur (18) observed that the addition of an inert polymer such as dextran sulphate increased the rate of hybridisation in solution. Thus the presence of 10% dextran sulphate gave rise to a 10-fold increase in reassociation rate. The effect was attributed to the exclusion of the DNA from the volume occupied by the polymer, that is, the dextran sulphate effectively increased the concentration of the DNA. A qualitatively similar effect occurs in filter hybridisation using both DNA and RNA probes (19) where, of course, the concentrating effect of the polymer applies only to the solution phase. For a single-stranded probe, the rate of hybridisation increases by 3- to 4-fold. For a double-stranded probe, the rate apparently increases by up to 100-fold and the yield of hybrid apparently also increases. However, in both cases, most of this increase is caused by the formation of concatenates which readily occurs under these conditions, that is, extensive networks of reassociated probe which, by virtue of single-stranded regions, hybridise to filter-bound nucleic acid and so lead to over-estimation of the extent of hybridisation. For qualitative studies this amplification in the hybridisation signal caused by binding of labelled probe is quite useful. However, for quantitative studies the effect may complicate the interpretation of results. Therefore it may be desirable to reduce the likelihood of networks forming by using probes which are not self-complementary. If double-stranded DNA probes are used they should be short to minimise the formation of extensive networks of probe. For example, if nick-translated, double-stranded DNA probes (Chapter 2, Section 4.1.2) are used, the DNase concentration in the nick-translation reaction should be adjusted to give fragments $\leq$400 nucleotides long. Short incubation times should also be used since the formation of networks occurs late in the reaction. Finally it should be noted that solutions of dextran sulphate are viscous (and so can be difficult to handle) and can lead to high backgrounds.

3.9 Mismatching

Many hybridisation reactions involve complex mixtures of sequences and the duplexes formed are not all perfectly base-paired. Mismatching has the effect of lowering the rate of hybridisation and the melting temperature of hybrids, T_m. The temperature dependence of k_i, the rate constant for the formation of mismatched hybrids, still gives a bell-shaped curve (Section 3.5), but k_i is lower and reaches its optimum at a lower temperature relative to the rate constant for formation of perfect hybrids (4,14). This has not been studied extensively, but available data suggest that if the reaction is carried out at a temperature which is optimal for the formation of mismatched sequences, that is, about 25°C below their T_m, the rate is reduced by a factor of two for every 10% mismatch (14).

3.10 Viscosity

As the viscosity of the solution increases, the rate of hybridisation decreases. The effect can be quite large, but there is insufficient data to formulate an exact relationship.

3.11 pH

The effect of pH has not been studied extensively but within the pH range 5 – 9, the rate of hybridisation at 0.4 M Na^+ is essentially independent of pH (ref. 12). In practice, hybridisation experiments are usually carried out at pH 6.8 – pH 7.4.

4. FACTORS AFFECTING HYBRID STABILITY

The melting temperature T_m is a measure of the thermal stability of hybrids. No systematic study of the effect of different parameters has been made for filter hybridisation. However, in general, variables that alter the rate constant, k, also alter the T_m in the same direction. The relationships below are derived from studies on hybridisation in solution, but are expected to be similar, qualitatively at least, for filter hybridisation. It is worth noting, however, that as a consequence of binding nucleic acid to the filters, the T_m of hybrids is often lower than would be predicted from solution hybridisation studies (5).

4.1 Perfectly-matched Hybrids

4.1.1 *DNA-DNA Hybrids*

Many studies on the stability of perfectly-matched DNA duplexes in solution have shown that T_m is dependent on ionic strength, base composition and denaturing agents (14, 20,21). The following relationship has been derived from combining several results (15):

$$T_m = 81.5 + 16.6 \text{ (log M)} + 0.41 \text{ (\%G+C)} - 0.72 \text{ (\% formamide)} \qquad \text{Equation 7}$$

where M is the molarity of the monovalent cation and (% G+C) is the percentage of guanine and cytosine residues in the DNA. The monovalent cation dependence holds between the limits of 0.01 – 0.4 M NaCl, but only approximately above this (20). The T_m is maximal at 1.0 – 2.0 M NaCl. The dependence on (% G+C) is valid between 30% and 75% (G+C) (ref. 22). The reduction in T_m by formamide is greater for poly(dA:dT) (0.75°C per 1% formamide) than for poly(dG:dC) (0.5°C per 1% formamide) (ref. 16).

In aqueous solution at 1 M NaCl (equivalent to 6 x SSC), Equation 7 simplifies to:

$$T_m = 81.5 + 0.41 \text{ (\% G+C)}$$

The following relationships, derived from solution hybridisation studies, are also useful:

(i) Every 1% mismatching of bases in a DNA duplex reduces the T_m by 1°C (ref. 14).

(ii) $(T_m)\ \mu_2 - (T_m)\ \mu_1 = 18.5 \log \mu_2/\mu_1$
where μ_1 and μ_2 are the ionic strengths of the two solutions (ref. 23).

4.1.2 *RNA-DNA Hybrids*

For RNA-DNA hybrids, the term in Equation 7 incorporating formamide concentration does not hold because the relationship between formamide concentration and the depression of T_m is not linear. At 80% formamide, RNA-DNA hybrids are more stable than DNA-DNA hybrids by some 10−30°C depending on the sequence (5,16). Carrying out the reaction in 80% formamide can therefore also be used to suppress formation of DNA-DNA duplexes and preferentially select RNA-DNA hybrids (5,16,24).

4.2 Mismatched Hybrids

The T_m of nucleic acid hybrids is depressed by base mismatching. Values obtained from solution hybridisation studies show that a 1% mismatch reduces the T_m by between 0.5 and 1.4°C (refs. 17,21,22,25,26). The exact figure depends on the (G+C) content of the DNA. The stability of the hybrids also depends on the distribution of mismatched bases in the duplex. Thus if two sequences have 20% base pair mismatch, the hybrid formed between them will have a high T_m if the mismatch is concentrated in one region leaving a long stretch of perfectly-matched duplex. In contrast, the hybrid will be extremely unstable if every fifth base is mismatched.

At high concentrations of salt, mismatched hybrids are more stable than at low concentrations. In practice this is very useful because varying the salt concentration can be used to stabilise or dissociate mismatched hybrids according to the requirements of the experiment.

5. DISCRIMINATION BETWEEN RELATED SEQUENCES

5.1 Stringency of hybridisation

A sizeable fraction of the eukaryotic genome is composed of families of similar, but not identical, sequences. It is often the aim of filter hybridisation studies to distinguish between closely- and distantly-related members of such a family, for example, in screening recombinant libraries or determining gene copy numbers by Southern blots (Chapter 5). In practice this means that reaction conditions must be adjusted to optimise hybridisation of one species and minimise hybridisation of others.

As explained in Section 3.5, bell-shaped curves describe the relationship between the rate of hybridisation and the temperature of incubation for formation of both well-matched and poorly-matched hybrids. For a poorly-matched hybrid, the rate constant is lower and the curve is displaced towards lower temperatures. When the ratio of rate constants (discrimination ratio) for cross-hybridisation and for self-hybridisation is plotted against temperature of reaction, a sigmoidal curve is obtained (*Figure 1*). At low temperatures, the ratio is high while at higher temperatures (approaching T_m −20°C for perfectly-matched hybrids), the ratio approaches zero (4,14). Although the data are not extensive, Beltz *et al.* (4) have suggested that this curve is probably a member of a family of sigmoidal curves whose exact dependence on temperature depends on the degree of mismatching of the hybrids. The relationship is useful in that it predicts that it should be easier to distinguish between distantly-related sequences by incubating at low temperatures while it should be easier to distinguish closely-related sequences by hybridising at high temperatures.

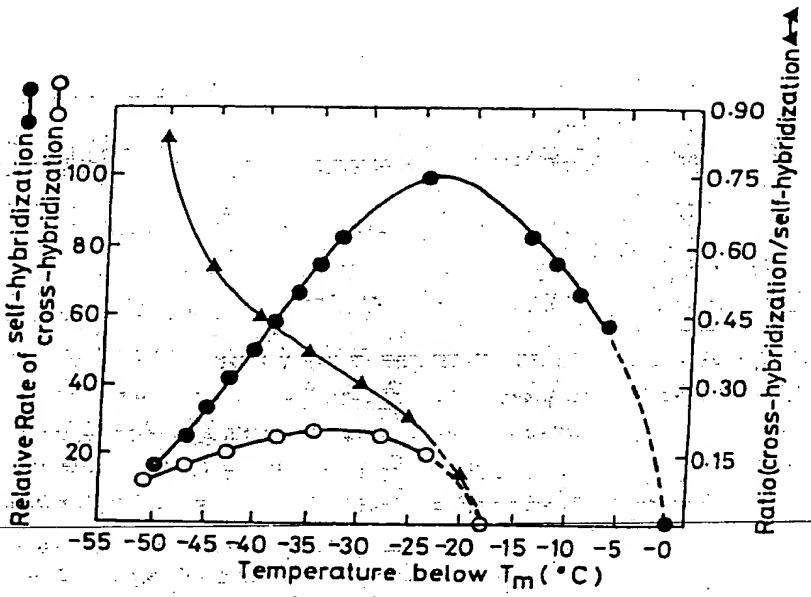

Figure 1. Rate of DNA reassociation as a function of temperature. Normal bacteriophage T4 DNA was used to examine the reassociation of perfectly-matched sequences (self-hybridisation; ●—●) and T4 DNA partially deaminated with nitrous acid was used for mismatched sequences (cross-hybridisation; ○—○). The dotted lines are extrapolations assuming that the rates of reassociation are zero at the appropriate T_m: for normal T4 DNA under these conditions (0.15 M sodium phosphate buffer) the T_m is 81°C. The discrimination ratio (▲—▲) is the rate constant for cross-hybridisation k_i, divided by the rate constant for self-hybridisation, k. Reproduced from reference 4 with permission.

In practice, therefore, to distinguish between the distantly-related members of a family of sequences, hybridisation should take place at a very permissive (relaxed) criterion. To detect closely-related members, the hybridisation should be at a stringent criterion. A single compromise criterion will not be effective because, as we have seen, different members of the family probably have different discrimination *versus* temperature curves. Hybridisation at a relaxed criterion followed by washing under progressively more stringent conditions may be useful for detecting distantly-related members of a family, but is not suitable for identifying closely-related members. This is probably because hybridisation and washing depend on different parameters. Hybridisation depends on the nucleation frequency while washing depends on the thermal stability (T_m) of the hybrids. Thus, a stringent hybridisation followed by a stringent wash is better for detecting closely-related members of a family than permissive hybridisation and a stringent wash.

5.2 Extent of Reaction

In distinguishing between related sequences, it is important to consider the extent of reaction. At first sight, it might appear that the longer the time of incubation the better should be the discrimination, but this is not the case. The following arguments have been made by Beltz *et al.* (4).

When two (or more) filter-bound sequences react with the same probe, the rate of

depletion of the probe is given by the following equation:

$$\frac{-dC_s}{dt} = k_1[C_f][C_s] + k_2[C_s]^2 + k_i[C_i][C_s] \qquad \text{Equation 8}$$

where $[C_s]$ here refers to the concentration of the probe in solution at time zero.

This equation is derived from Equation 1 by the addition of a term to allow for cross-reaction of the probe with a related, filter-bound sequence, i, which has a concentration $[C_i]$ and a hybridisation rate constant k_i. $[C_f]$ is the concentration of filter-bound sequence, f, which is identical to the probe. The kinetics differ considerably depending upon which sequences are in excess and whether the probe can reassociate. For simplicity, in the following analyses (Sections 5.2.1 – 5.2.3) we have assumed that the concentrations of filter-bound sequences are the same (that is $[C_f] = [C_i]$) and $k_1 = k_2$. In fact we know that the rate constants are not equal, but the result will be qualitatively the same.

5.2.1 *Filter-bound Nucleic Acid in Excess*

When the filter-bound sequences are in excess over the probe, as in typical plasmid DNA dot blots (Section 6.2.1), Equation 8 simplifies to a pseudo-first order reaction where the rate of loss of the probe ($-dC_s/dt$) is given by:

$$\frac{-dC_s}{dt} = k_i[C_f][C_s] + k_i[C_i][C_s] \qquad \text{Equation 9}$$

Since $[C_f] = [C_i]$

then $$\frac{-dC_s}{dt} = [C_f]\,[C_s]\,\Sigma\, k$$

The rate of hybridisation to sequence i equals $[C_f][C_s]k_i$.
Therefore,

$$\frac{\text{rate of hybridisation to sequence i}}{\text{rate of hybridisation to all sequences}} = \frac{[C_f][C_s]k_i}{[C_f][C_s]\Sigma k} \qquad \text{Equation 10}$$

$$= \frac{k_i}{\Sigma k}$$

In a hybridisation reaction, it is more likely that there will be a number (m) of cross-reacting species. The overall reaction can be treated as the sum of a number of independent hybridisations each with a different rate constant and each following pseudo-first order kinetics. When they go to completion, the probe will all be in hybrids. The frac-

tion of the probe hybridised to sequence i is given by:

$$\frac{k_i}{\sum_1^m k}$$

At any time during the reaction, the ratio of the amounts of probe hybridised to sequence i and to any other filter-bound sequence j is given by the ratio of the rate constants (k_i/k_j) and the ratio is not affected by the time of incubation. Hence the discrimination between related hybrids is not affected by the extent of the reaction because all the filter-bound sequences continuously compete for the same limiting probe (*Figure 2*).

5.2.2 *Single-stranded Probe in Excess*

The kinetics of hybridisation are different from that described above when the probe is in excess over the filter-bound sequences, as in typical genomic Southern blots, genomic dot blots, RNA dot blots and Northern blots (Chapters 5 and 6). If the probe is single-stranded and so cannot reassociate (e.g., for M13 or SP6 RNA probes), Equation 8 simplifies to the same form as Equation 9:

$$\frac{-dC_s}{dt} = k_1[C_f][C_s] + k_i[C_i][C_s]$$

If $[C_f] = [C_i]$, then rearranging terms,

$$\frac{-dC_s}{dt} = [C_s] \sum_{i=1}^{i} k_i[C_i]$$

It can be shown that $E_i(t)$, the fraction of filter-bound sequence i actually hybridised at time t, is given by the equation:

$$E_i(t) = 1 - e^{-k_i[C_s]t} \qquad \text{Equation 11}$$

and the ratio of the extent of hybridisation of cross-hybridising sequence i, to perfectly-matched sequence f is:

$$\frac{E_i(t)}{E_f(t)} = \frac{1 - e^{-k_i[C_s]t}}{1 - e^{-k_1[C_s]t}} \qquad \text{Equation 12}$$

This ratio is not constant but varies with time. The discrimination (that is, the actual extent of cross-hybridisation compared with the hybridisation to perfectly-matched sequences) is maximal very early in the reaction when it equals k_i/k_1, but declines with increasing incubation time as the term at the right hand side of Equation 9 approaches unity (*Figure 2*). This means that although the homologous reaction is faster and will

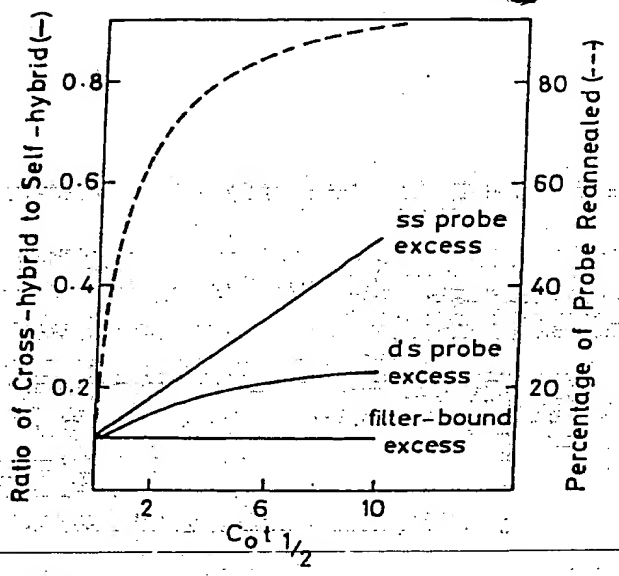

Figure 2. Effective discrimination between perfectly-matched and mismatched sequences as a function of the extent of the reaction. The solid lines represent the ratio $[E_i(t)/E_f(t)$, see text] of the amounts of probe hybridised to filter-bound mismatched, heterologous sequences (cross-hybridisation) and perfectly-matched, homologous sequences (self-hybridisation). Three separate reactions are shown: filter-bound sequences in excess; denatured double-stranded DNA probe in excess; single-stranded probe in excess. The discrimination ratio (k_i/k) is assumed to be the same (0.1) in all cases. The dashed line shows the normal kinetics of reassociation of denatured double-stranded DNA in solution. Reproduced from reference 4 with permission.

reach completion earlier, the heterologous reaction will eventually catch up (single-stranded probe excess; *Figure 2*). In practical terms, then, with increasing time of reaction, discrimination becomes poorer, so reaction times should be kept short.

5.2.3 *Double-stranded Probe in Excess*

Consider Equation 8 again:

$$\frac{-dC_s}{dt} = k_1[C_f][C_s] + k_2[C_s]^2 + k_i[C_i][C_s] \qquad \text{Equation 8}$$

If the probe can reassociate, the term $k_2[C_s]^2$ is significant. Therefore, as the reaction proceeds, reassociation in solution reduces the amount of probe that is available to hybridise to the filter-bound sequences. So the reaction changes from being in probe excess to one in which filter-bound nucleic acid is in excess. It can be shown that:

$$\frac{\text{fraction of probe hybridising to sequence i}}{\text{fraction of probe hybridising to perfectly-matched sequence f}} = \frac{E_i\ (t)}{E_f\ (t)}$$

$$= \frac{1 - (1+k_2[C_s]t)^{-n}}{1 - (1+k_2[C_s]t)^{-1}} \qquad \text{Equation 13}$$

where n = k_i/k_1, that is, the discrimination ratio. Again this means that in practical terms, discrimination equals k_i/k_1 very early in the reaction, but deteriorates rapidly. So, in practice, to distinguish between cross-hybridising species, it is best to use short times of incubation regardless of whether the probe or the filter-bound sequence is in excess. If this does not generate enough signal to be detected, it is advisable to use excess filter-bound sequence and to hybridise for longer times.

6. BINDING OF NUCLEIC ACID TO FILTERS

6.1 Types of Filter Material

There are several types of filter currently in use for the immobilisation of DNA and RNA, for example, nitrocellulose, nylon and chemically-activated papers. The material of choice depends on the purpose of the experiment.

Nitrocellulose filters bind DNA and RNA very efficiently, (~ 80 μg/cm^2) except for small fragments of less than about 500 nucleotides in length which are bound rather poorly. The binding procedure is simple. The main disadvantage of nitrocellulose is that it is rather fragile so it requires careful handling and on repeated use tends to become brittle and fall apart. Nylon filters are more pliable than nitrocellulose, are easier to handle and can be used indefinitely without disintegrating. They are reputed to bind nucleic acid as efficiently as nitrocellulose and on the whole we have found that this is true, but we have experienced batch variation in the binding efficiency of some brands. For Southern, Northern and dot blots, both nitrocellulose and nylon filters give excellent results. For DNA dot blots, filters with a pore size of 0.45 μm are used for large nucleic acid molecules and 0.22 μm for molecules of less than 500 nucleotides. For RNA dot blots, filters with 0.1$-$0.22 μm pore size are most efficient.

Although nitrocellulose and nylon filters immobilise nucleic acid, binding by conventional procedures is not covalent. This can lead to problems. For example, nucleic acid is gradually leached off the surface when filters are hybridised for long periods, particularly at high temperature. Furthermore, if the probe in solution is complementary to the entire length of the filter-bound sequence, the hybrid dissociates from the filter and is lost into solution (27). So a consequence of non-covalent binding of nucleic acid is that the hybridisation sensitivity may be reduced with time. New techniques have now been developed for covalent binding of nucleic acid to membranes. This involves u.v. light-induced binding (28). However, to date this is only applicable to nylon filters because of the risk of fire when using nitrocellulose membranes. Chemically-activated paper binds nucleic acid covalently so it has the advantage that it does not discriminate against small nucleic acid molecules and does not lose nucleic acid sequences once they are bound. Both cellulose and nylon filters can be chemically activated. However, the binding capacity of chemically-activated paper (1$-$2 μg/cm^2) is much lower than other types of filter and the binding procedure is more complicated. Hence it is not much used for Southern, Northern and dot blots; its main use is in hybrid selection for the enrichment of specific RNA sequences (Chapter 5, Section 3.2).

Suppliers of nitrocellulose filters are Schleicher and Schüll (membrane filters BA85), Millipore U.K. and Waters Associates (Millipore filters), Sartorius Instruments Ltd. (Sartorius filters) and Amersham International plc (Hybond C filters). Suppliers of nylon filters are New England Nuclear (GeneScreen and GeneScreen Plus hybridisation transfer

membrane), PALL (Biodyne transfer membrane) and Amersham International plc (Hybond-N membranes). Clearly, this list can never be complete since new products are continually being marketed. The filters are generally available as circles and rectangles in several sizes. Most are also available in rolls which can be cut to size.

All filters require the nucleic acid to be denatured for binding. However, it is most important to note that there is no immobilisation procedure uniformly applicable to all types of filter. Nitrocellulose filters require high ionic strength for quantitative binding of both DNA and RNA and the binding efficiency is much reduced at low ionic strength (29,30). In contrast, GeneScreen nylon membranes require low ionic strength for binding and the binding is poor at high salt concentrations. We have successfully used the same procedures for binding to nitrocellulose and to Biodyne nylon membranes.

6.2 DNA Dot Blots

Multiple samples of genomic or plasmid DNA are spotted next to each other on a single filter in dots of uniform diameter. For quantitative analysis, known amounts of DNA are applied. To evaluate the extent of hybridisation of the probe, a standard consisting of a dilution series of DNA dots is applied in an identical way to the same filter. The procedure binds samples quickly so that many samples can be handled at once. As little as 1 – 3 pg of a hybridising DNA sequence can be detected. Dot blots do not distinguish the number and size of the molecules hybridising, so the hybridisation 'signal' is the sum of all sequences hybridising to the probe under the conditions used.

Commercial apparatus has been developed for binding multiple samples of DNA to filters. A protocol for use of this is described elsewhere in this volume (Chapter 5, Section 3.1). Not every laboratory has access to such a device so the procedure described here involves manual application of samples. This is more time consuming and the dots are less uniform than when applied by the multiple filtration device, but the results are perfectly satisfactory.

There are many protocols in use for binding samples to filters. They can be divided into two classes according to whether the DNA is denatured before or after it is applied to the filter. Both give satisfactory results. An example of the former method is described for use with the multiple filtration device (Chapter 5, Section 3.1.2). We shall describe an example of the latter method which is in current use in our laboratory with nitrocellulose and Biodyne filters. It is not applicable to GeneScreen filters which require low salt concentration for DNA binding.

6.2.1 *Plasmid DNA Dot Blots*

It is necessary to convert supercoiled DNA to open circular or linear form to bind to filters. This is because DNA must be single-stranded for binding and denatured supercoiled DNA renatures too quickly on neutralisation to be trapped in the denatured state. Two common ways of obtaining linear or nicked plasmid DNA are to restrict the DNA by enzymic digestion and to treat the plasmid at high temperature (see *Table 1*). The latter partially depurinates the DNA so that on subsequent treatment with alkali the phosphodiester bond breaks at the site of depurination (31). Linear DNA will then separate into single strands.

Nitrocellulose filters are usually treated with high concentrations of salt either at the

Table 1. Linearisation of Plasmid DNA.

Restriction Method

1. Digest the recombinant plasmid with a suitable restriction enzyme. Monitor linearisation of the plasmid by agarose gel electrophoresis.
2. Extract the restricted DNA with an equal volume of phenol pre-saturated with 10 mM Tris-HCl, pH 7.5, 0.1 mM NaCl, 1 mM EDTA. Spin for 2 min in a microcentrifuge to separate the layers.
3. To the aqueous phase, add 0.5 vol. of 7.5 M ammonium acetate and precipitate the DNA by adding 2.5 vol. ethanol pre-cooled to −20°C. Mix well and place at −20°C overnight or −70°C for 1−2 h.
4. Recover the DNA by centrifugation and dry briefly under vacuum.
5. Resuspend the DNA at 50 μg/ml in TE buffer, pH 8.0[a].

High Temperature Method

1. Place 20 μg DNA in a microcentrifuge tube in a final volume of 100 μl 20 mM Tris-HCl, pH 7.4, 1 mM EDTA.
2. Pierce the lid to prevent it popping open and place the tube in a boiling water bath for 10 min.
3. Chill in ice and centrifuge for 10 sec to ensure that all the sample is at the bottom of the tube.
4. Check the volume and adjust to 100 μl with water if necessary so that the DNA concentration remains at 20 μg/100 μl.

[a]TE buffer is 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.

same time as, or prior to, binding of nucleic acid. This both improves the efficiency of binding and helps to keep the diameter of the dot small. Salts commonly used are 1 M ammonium acetate or 20 x SSC [1 x SSC is 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0]. A suitable procedure for binding plasmid DNA to nitrocellulose filters involving pre-treatment of the filter with high salt is given below. It is important to note that the filter must not at any stage be handled with bare hands. Grease from the fingers will result in poor binding of nucleic acid and high backgrounds. Therefore disposable plastic gloves must be worn at all stages.

(i) Float a sheet of nitrocellulose on water taking care not to trap air bubbles underneath. When one side is wet, immerse the filter completely to wet the other. If there are dry patches which are reluctant to wet, boil the water for a few minutes.

(ii) Blot the filter lightly on Whatman 3MM paper and transfer to a dish containing 20 x SSC. Leave for 30 min with gentle shaking.

(iii) Dry at room temperature or under a lamp until completely dry.

(iv) If convenient, use a conventional rubber stamp and ink pad to stamp the paper with an array of 5 mm diameter circles to allow easy identification of samples. At this stage, the filters can be stored dry at room temperature sealed in a polythene sleeve.

(v) Place a nitrocellulose filter which has been treated with 20 x SSC onto the lid of a plastic box such that only the edges of the filter are in contact with the lid.

(vi) Apply the plasmid (0.8 μg in 4 μl, linearised as in *Table 1*) to the filter. This can be achieved using a 1−5 μl Supracap pipette (Brand) or an automatic Pipetman (Gilson). Be careful not to puncture the nitrocellulose filter. Keep the diameter of the dots small and do not exceed 4 mm. If necessary, make repeated applications allowing time for each application to dry.

(vii) Allow the samples to dry at room temperature or under a lamp.

Table 2. Reducing the Size of Eukaryotic DNA Prior to Filter Binding.

1. *Either* sonicate 30 μg DNA to an average size of 2 kb or restrict it with a suitable restriction enzyme and then remove the enzyme by phenol extraction (*Table 1*, step 2). The size of the DNA should be checked by agarose gel electrophoresis using appropriate size markers (Appendix II).
2. Recover the DNA by ethanol precipitation (*Table 1*, step 3).
3. Wash the DNA in 70% ethanol, dry briefly under vacuum and resuspend in 1 ml TE buffer, pH 8.0[a].
4. Measure the concentration of DNA spectrophotometrically using the conversion factor $A_{260nm} = 1$ for a solution of 50 μg/ml.
5. Check the volume of solution and freeze dry.
6. Resuspend the DNA in water at a concentration of 10 μg per 4 μl. For serial dilutions, prepare a set of microcentrifuge tubes each containing 6 μl TE buffer, pH 8.0. Remove 6 μl DNA into the first tube containing TE buffer. Mix well. Remove 6 μl from this tube into the second tube with TE buffer and so on until seven dilutions have been made (10 μg–5.3 ng per 4 μl).
7. Centrifuge briefly in a microcentrifuge to ensure that each DNA solution is at the bottom of the tube.
8. Apply the samples to a dry sheet of nitrocellulose pre-treated with 20 x SSC as described in Section 6.2.1, steps (v) – (xii).

[a]TE buffer is 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.

(viii) Denature the DNA by placing the filter, application side up, on a sheet of Whatman 3MM paper saturated, but not 'swimming', in 1.5 M NaCl, 0.5 M NaOH. Leave for 5 min. (This is conveniently done in a plastic tray.)

(ix) Transfer the filter to Whatman 3MM paper saturated with 0.5 M Tris-HCl, pH 7.4, for 30 sec.

(x) Transfer the filter to Whatman 3MM paper saturated in 1.5 M NaCl, 0.5 M Tris-HCl, pH 7.4, for 5 min. The DNA is now reversibly bound to the filter.

(xi) Place the filter on a dry sheet of 3MM paper and leave to dry at room temperature.

(xii) Sandwich the filter between two sheets of 3MM paper and bake at 80°C for 2–3 h to immobilise the DNA. Ideally, nitrocellulose filters should be baked in a vacuum oven to reduce the risk of fire.

6.2.2 *Genomic DNA Blots*

Like plasmid DNA, genomic DNA can be applied to filters in dots. The amount of DNA required per dot depends on the experiment being performed. To detect a single copy sequence in eukaryotic DNA, a minimum of 10 μg DNA per dot is suggested. For sequences present in multiple copies, proportionately less can be used.

Modern techniques for DNA isolation usually give a product which is very concentrated and has a very high molecular weight. These two factors make the DNA solution very viscous so that it is difficult to measure the concentration accurately. Hence, to do this, it is necessary to reduce the size of the DNA either by sonication or digestion with an appropriate restriction endonuclease (*Table 2*). This also helps to bind the DNA to the filter more efficiently. Binding of the DNA to nitrocellulose filters, pretreated with 20 x SSC, is carried out as described above for plasmid DNA (Section 6.2.1).

6.3 RNA Dot Blots

The principle of this procedure is exactly the same as for DNA dot blots. Known amounts of RNA are applied to an inert support and the amount of specific RNA sequence is

determined by hybridisation with a suitable labelled probe. Evaluation of the extent of hybridisation can be made by comparison with standards. The technique is sensitive — as little as 1 pg of a specific RNA sequence can be detected (32). As with DNA dot blots, however, the procedure gives no information on the size or number of sequences contributing to the hybridisation signal. Nylon and nitrocellulose filters are suitable supports. Because nitrocellulose tends to be the most used, its use will be described here. Chemically-activated paper is not generally used for RNA dot blots as its binding capacity is too low.

6.3.1 *Preliminary Precautions*

One of the main problems of working with RNA is its extreme sensitivity to degradation. Glassware must be scrupulously clean and should never be touched with bare hands which are a good source of ribonuclease. Prior to use, the glassware should be treated with diethylpyrocarbonate to inactivate any ribonuclease. This can be done by immersing the glassware in water to which has been added two drops per litre of diethylpyrocarbonate and then boiling for 30 min. The glassware can then be dried. Heavy metal ions can lead to degradation of RNA especially when long incubations are involved so these should be removed by filtration of all solutions through Chelex resin (BioRad) before use.

6.3.2 *Denaturation of RNA and Binding to Filters*

Although RNA is single-stranded, it contains double-stranded regions which must be denatured for efficient binding to filters. Alkali treatment is not suitable since it degrades RNA and heat denaturation has not been found to give efficient binding (32). Commonly used denaturants for RNA include glyoxal (33), methyl mercuric hydroxide (34), formaldehyde (35) and dimethyl sulphoxide (DMSO) (36). Since methyl mercuric hydroxide and formaldehyde are toxic and DMSO dissolves nitrocellulose, the procedure described here uses glyoxal as a denaturant. It is based on the methods developed by Thomas (32,37). Glyoxal is supplied commercially as a 40% aqueous solution (6.89 M) which contains polymerisation inhibitors. Glyoxal is readily oxidised to glyoxilic acid which degrades RNA so it is necessary to purify the glyoxal before treating the RNA. This is usually done by deionisation. A suitable protocol is as described in Chapter 6, *Table 1*.

Glyoxal denatures RNA (and DNA) by binding covalently to guanine residues forming an adduct which is stable at acid and neutral pHs. Glyoxylated nucleic acid binds efficiently to nitrocellulose paper but, after binding, the glyoxal groups must be removed because they have an inhibitory effect on hybridisation. This is easily and quantitatively achieved by treating the filter at 100°C at pH 8.0. Under these conditions as little as 1 pg of a specific sequence of RNA can be detected. The detailed procedure for glyoxalation of RNA and binding to nitrocellulose filters is as follows.

(i) Dry down 20 μg of the sample RNA in a microcentrifuge tube and dissolve it in 5 μl water. The RNA should be salt-free and free of protein which will otherwise react with the glyoxal.

(ii) Prepare a denaturation solution: 34 μl deionised glyoxal, 20 μl 0.1 M sodium phosphate buffer, pH 6.5, 46 μl water.

(iii) Add 5 μl denaturation solution to the RNA. Cover the tube and incubate for 1 h at 50°C. (If using a water bath, make sure that the water comes well up the sides of the tube in order to minimise evaporation.)

(iv) Centrifuge in a microcentrifuge (10 sec) to ensure the sample is at the bottom of the tube. After denaturation in glyoxal, the samples are stable for a few hours and can be kept at 4°C.

(v) If required, make serial dilutions as described in *Table 2* (step 6) but using 1% SDS as the diluent.

(vi) Apply the samples by hand to a sheet of nitrocellulose [pre-treated in 20 x SSC as described in Section 6.2.1, steps (i)—(iv)] using the application procedure described in Section 6.2.1, steps (v) and (vi).

(vii) Bake the filter at 80°C for 2 h to immobilise the RNA.

(viii) Remove the glyoxal groups by placing the filter in water at 100°C for 5—10 min, then allowing the water to cool to room temperature.

(ix) Blot the filter on 3MM paper and allow to dry at room temperature or under an infra-red lamp.

7. NUCLEIC ACID PROBES

7.1 Types of Probe

In theory, any nucleic acid can be used as a probe provided that it can be labelled with a marker which allows identification and quantitation of the hybrids formed. In practice, double- and single-stranded DNAs, mRNA and RNAs synthesised *in vitro* are all used as probes. Oligonucleotide probes are not used in quantitative dot blots; they are most useful for screening recombinant DNA libraries.

7.1.1 *Double-stranded DNA Probes*

Double-stranded DNA probes are very commonly used in dot blot analysis. They are often cloned sequences and have low complexity. There are two important points to note when using double-stranded DNA probes:

(i) Two competing reactions occur in filter hybridisation, *viz.* reassociation of the probe in solution and hybridisation to the filter-bound nucleic acid. Therefore, reaction conditions should be chosen to optimise the latter (see Section 11).

(ii) If the DNA is a cloned sequence, it should be excised and purified away from the vector. This avoids complications which can arise if single-stranded vector tails allow formation of concatenates in solution, particularly if the DNA has been randomly sheared. Furthermore, if filters are re-hybridised and the previous probe containing vector sequences has not been completely removed, sandwich hybridisation may occur. That is, duplexes may form through vector sequences rather than through insert sequences. This complicates the interpretation of results.

7.1.2 *Single-stranded DNA Probes*

With single-stranded DNA probes there is no competing reassociation in solution so filter hybridisation is favoured and reactions can be carried out for longer. Single-stranded DNA probes are obtained by strand separation of double-stranded DNA (see Chapter 6, Section 4.2.4) or from M13 phage recombinants.

7.1.3 *RNA Probes*

RNA probes are more difficult to handle than DNA probes because of the widespread presence of ribonucleases. In addition, mRNA probes, or cDNAs derived from them, are often complex mixtures of sequences and therefore the sequence of interest may represent only a very small proportion of the total nucleic acid. Since the rate of filter hybridisation is inversely proportional to the complexity for low amounts of filter-bound nucleic acid, it may be difficult or impossible to detect the desired hybrids.

Recently it has proved possible to synthesise large amounts of RNA *in vitro* from specially-constructed recombinant plasmids, such as the SP6 plasmids. The probes have low complexity and, because they are single-stranded, there is no competing reassociation reaction in solution. For these reasons, the use of SP6 RNA transcripts as probes is proving increasingly popular.

7.2 Radiolabelled Probes

Traditionally, filter hybridisations have been carried out with radioactively-labelled probes. ^{32}P is the most commonly used radionuclide and will be the only one discussed here. Conventional labelling replaces a proportion of the nucleotides in a nucleic acid with ^{32}P derivatives or adds ^{32}P to the end of the molecule. After hybridisation, hybrids are detected by autoradiography. ^{32}P has the advantage over other radioisotopes that high specific activities can be readily attained. Much of the technology of filter hybridisation has been developed with it. However, precautions must be taken when handling ^{32}P because of the radiation emitted. Detection by autoradiography, while sensitive, may take a long time if there are few counts in the hybrids. Furthermore, since ^{32}P has a half-life of 14.3 days, experiments should be completed within one half-life.

The preparation of radioactively-labelled DNA and RNA probes, including SP6 RNA transcripts, is described in detail in Chapter 2. In preparing labelled probes for filter hybridisations it is important to remove unincorporated precursors efficiently before use (see Chapter 2) otherwise they may bind non-specifically, but irreversibly, to the filter, giving a high background.

7.3 Non-radioactive Probes

Recent advances in nucleic acid technology now offer alternatives to radioactively-labelled probes. For example, single-stranded DNA can be coupled to a protein. If this protein-DNA complex is now hybridised to filter-bound nucleic acid, the protein in the duplex can be visualised by an antibody reaction (38). If the protein is an enzyme such as peroxidase, then it can be detected and quantitated by its ability to convert a colourless substrate into an insoluble coloured pigment at the site of hybrid formation. This technique is sensitive (1 – 5 pg nucleic acid can be detected) and has some potentially useful applications. For example, DNA probes coupled to different enzymes can be used in the same hybridisation reaction, so that it should be possible to detect the presence of unrelated sequences simultaneously (38).

Another procedure that uses non-radioactive probes and is becoming increasingly popular is biotin labelling of nucleic acid (39,40). These probes are prepared in a nick-translation reaction by replacing nucleotides with biotinylated derivatives. After hybridisation and washing, detection of hybrids is by a series of cytochemical reactions which

finally give a blue colour whose intensity is proportional to the amount of biotin in the hybrid. Biotinylated probes detect target sequences with the same sensitivity as radioactive probes, that is, in the 1–5 pg range. There are several advantages of using biotinylated probes. For example, non-toxic materials are employed and there are no problems of inconveniently short half-lives of the label. This has the additional bonus that biotin-labelled probes can be prepared in advance in bulk and stored at −20°C until required. Detection of hybrids is much faster than for radioactive probes, visualisation of hybrids being complete 2–4 h after washing. One disadvantage of biotin-labelled probes is that the cytochemical visualisation reactions lead to precipitation of insoluble material which cannot be removed, so when the filter is re-used, the previous 'signals' are still present (39,40). The preparation and use of non-radioactive nucleic acid probes is discussed in Chapter 2, Section 4.3.

7.4 Additional Considerations

Additional factors which should be borne in mind when choosing probes are:

(i) It is important to characterise the nucleic acid used for the probe. If any repetitive elements are present (e.g., *Alu*I sequences), they must be removed if the probe is to be used to detect low copy number sequences otherwise hybridisation of the latter will be masked by the repetitive sequence hybridisation.

(ii) The length of the labelled probe is important since the kinetics of hybridisation depend on probe length (see Section 3.3).

(iii) The kinetics of hybridisation differ according to whether the probe or filter-bound sequences are in excess and it is not always immediately apparent which is in excess. What is important is the concentration of the hybridising species, not the total nucleic acid concentration. The following is a rough guideline to this problem. With genomic Southern and Northern blots and genomic DNA and RNA dot blots, the concentration of the probe is likely to be in excess. For example, in *Figure 3*, even though there is 10 μg RNA per dot and the Ha-*ras*1 probe is at 20 ng/ml, the probe is in excess for RNA taken from normal tissue. (However, note that the filter-bound sequences are in excess for RNA taken from diseased tissue.) With plasmid or phage dot blots, and phage and colony screening, the filter-bound sequences are likely to be in excess. However, to check which is in excess, the following preliminary experiments can be performed.
   (a) Vary the input of probe; if the filter-bound sequence is in excess, the amount of hybridisation should be proportional to the probe input.
   (b) Vary the amount of nucleic acid on the filter; if it is in excess, there should be no difference in the amount of probe hybridised.

8. HYBRIDISATION USING RADIOACTIVE PROBES

8.1 Choice of Reaction Conditions

There are many protocols available for hybridising a probe in solution to nucleic acid immobilised on filters. The conditions used depend on the purpose of the experiment and in general are governed by whether DNA-DNA or DNA-RNA hybridisation is involved and whether closely-related or distantly-related sequences are reacting. Reaction conditions that permit formation of hybrids which have a high degree of mismat-

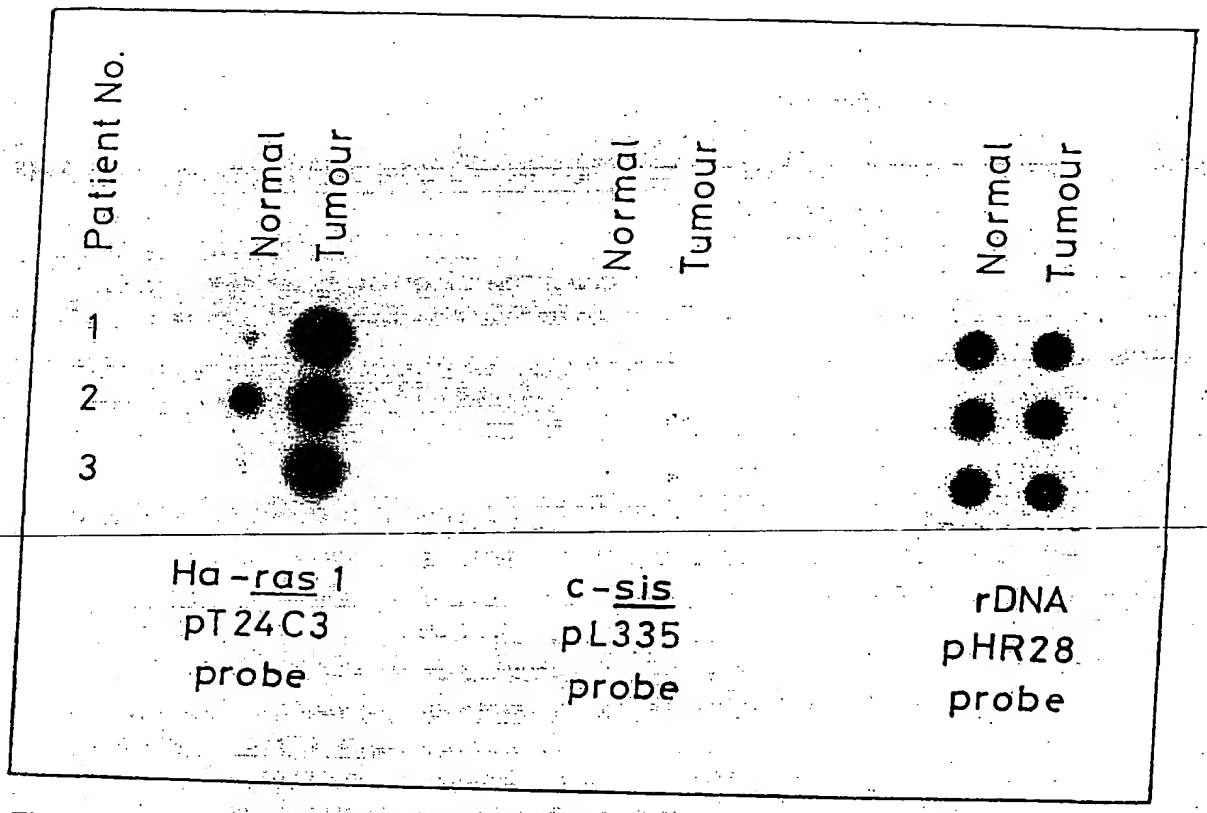

Figure 3. RNA dot blots. Replicate filters containing 10 μg per dot of poly(A)$^+$ RNA from normal and tumour breast tissue of three different patients were hybridised with the following denatured ^{32}P-labelled double-stranded probes: pT24C3 (Ha-*ras*-1), pL335 (c-*sis*) and pHR28 (rDNA). Equal amounts of RNA were present in the dots as judged by the intensities of the hybridisation signals using the rDNA probe. Transcripts homologous to the Ha-*ras*-1 probe are more abundant in tumour tissue compared with normal breast tissue and this difference is specific to the *ras* oncogene since the c-*sis* probe did not give a signal. (Data of Agnatis and Spandidos, with permission.)

ching are said to be permissive (relaxed or low stringency), while those which allow only well-matched hybrids to form are said to be stringent (high stringency). However, the same basic procedure is followed irrespective of the particular reaction conditions used. We will first describe standard hybridisation protocols which have widespread applicability (Sections 8 – 10) and then how varying the reaction conditions can be exploited to detect different hybrids (Section 11).

The hybridisation process can be divided into three steps: pre-hybridisation, hybridisation with a labelled single-stranded probe, and washing. In the *pre-hybridisation* step, the filter is incubated in a solution which is designed to pre-coat all the sites on it which would bind the probe non-specifically. Failure to do this leads to high backgrounds. Typically, the solution contains Ficoll, polyvinyl pyrollidone and bovine serum albumin [i.e. Denhardt's solution (ref. 41)], and heterologous DNA. As an alternative, heparin can substitute for Denhardt's solution (42). To reduce backgrounds even further, poly(A) and poly(C) are often included. Poly(A) is useful when the probe or filter-bound sequences are rich in A and T residues, e.g., poly(A)$^+$ mRNA or cDNA derived from it. Similarly, poly(C) is included if the probe or filter-bound sequences are rich in G and C residues as when a recombinant is generated through oligo(dG) and oligo(dC)

homopolymer tailing. For hybridisations involving RNA, yeast tRNA is often used as a competitor. For *hybridisation*, it is necessary to ensure that the added nucleic acid probe is single-stranded. For double-stranded DNA probes this is usually achieved by boiling or by denaturing in alkali. Radioactive probes can be denatured using either method; heat denaturation is described here and alkaline denaturation is described in Chapter 5 (*Table 3*, step 6). For most purposes hybridisation can be carried out in either aqueous solution or in the presence of formamide. We use the same formamide-containing solutions for both RNA-DNA and DNA-DNA hybridisations, but aqueous solutions for DNA-DNA hybridisations only. Both protocols can be used with nitrocellulose and nylon filters and are described below. After hybridisation, *washing* is carried out to remove unhybridised probe and to dissociate unstable hybrids. The temperature and salt concentration of the washing solution determine which hybrids will be dissociated. In general, washing should be under as stringent conditions as possible; at 5 – 20°C below T_m for well-matched hybrids and 12 – 20°C below T_m for cross-hybridising species. In practice, 65 – 70°C is usually chosen for hybrids having a high degree of homology and 50 – 60°C for poorly-matched hybrids.

Where possible, the pre-hybridisation, hybridisation and washing steps should be carried out in a shaking water bath or on a shaking platform in an incubator. In filter-bound nucleic acid excess, diffusion of the probe to the filter can be limiting in the absence of agitation. Also, high backgrounds are sometimes encountered if there is no shaking. Solutions should be pre-warmed to the required temperature prior to use.

8.2 Hybridisation in the Presence of Formamide

8.2.1 *Pre-hybridisation*

(i) To wet the filters evenly, float them on a solution of 1% Triton X-100, taking care to prevent air bubbles being trapped underneath. When one side is wet, immerse the filter to wet the other side.

(ii) Remove the filters and blot gently on Whatman 3MM paper to remove excess liquid.

(iii) It is convenient to carry out the pre-hybridisation and hybridisation reactions in the same container. Typically this is a polythene bag. Suitable bags are 'Sears Seal-N-Save Boilable Cooking Pouches' or Layflat polythene tubing (Trans-Atlantic Supplies). Place each wet filter in a separate bag and heat-seal this, except for one corner, using a domestic bag sealer.

(iv) Add the pre-hybridisation solution (0.08 ml/cm² of filter) which has been prepared as described in *Table 3* and pre-warmed to 42°C. Gently squeeze out the air bubbles and heat seal the corner.

(v) Incubate the filter in the bag for 4 – 24 h at 42°C. This can be done by placing the bag in a box of water at 42°C in a shaking water bath at the same temperature. Set the water bath to shake at a speed such that the liquid in the bag sweeps gently over the surface of the filter. Alternatively, place the bag on a shaking platform in an incubator at 42°C.

(vi) Cut a corner of the bag and drain the liquid out. Roll a pipette over the surface of the bag to remove as much of the liquid as possible. However, it is most

Table 3. Preparation of Pre-hybridisation Buffer Containing Formamide.

Solution A	
Mix together:	
Deionised formamide[a]	50 ml
20 x SSC[b]	25 ml
100 x Denhardt's solution[c]	5 ml
1 M sodium phosphate buffer, pH 6.8[d]	5 ml
20% SDS[e]	0.5 ml
Adjust the volume to 95.5 ml with water.	
Solution B	
Mix together:	
Sonicated calf thymus DNA or salmon sperm DNA at 5 mg/ml[f]	2 ml
Poly(C) [5 mg/ml][g]	0.2 ml
Poly(A) [5 mg/ml][g]	0.2 ml
Yeast tRNA (5 mg/ml)[g]	2 ml
Denature in a boiling water bath for 5 min.	
Quench in ice.	
Add solution B to solution A and store at 4°C.	

[a]Formamide is a teratogen. Handle with care and use gloves. All contaminated glassware should be soaked overnight in dilute H_2SO_4 then rinsed with water before washing as usual. To deionise formamide, add 200 ml formamide to ~10 g of AG501-X8(D) mixed-bed resin (Bio-Rad). Stir for 1 h at room temperature. Filter through Whatman No. 1 filter paper to remove the resin. Store at 4°C in a dark bottle.
[b]The composition of SSC is 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0.
[c]100 x Denhardt's solution contains 2% Ficoll (mol. wt. 400 000), 2% polyvinyl pyrrolidone (mol. wt. 400 000) and 2% bovine serum albumin. Store at −20°C.
[d]1 M sodium phosphate buffer, pH 6.8, is made by mixing 25.5 ml of 1 M NaH_2PO_4 and 24.5 ml of 1 M Na_2HPO_4. Store at room temperature.
[e]Store this stock solution at room temperature.
[f]Add the DNA to water at ~5 mg/ml. Stir; it may take several hours for the DNA to dissolve. Then sonicate to a length of 400−800 bp. The size can be checked by agarose gel electrophoresis. Adjust the concentration to 5 mg/ml [A_{260nm} = 1 for a solution of 50 μg/ml]. Store at −20°C.
[g]These solutions are stored at −20°C. Their addition to the pre-hybridisation buffer is optional (see Section 8.1). However, there is no disadvantage in adding them even if T- and G-rich sequences are not present in the filter-bound nucleic acid sequence.

important that the filter is not allowed to dry out if high backgrounds are to be avoided. Therefore the filters should be left in the pre-hybridisation buffer in the bag until just before applying the hybridisation solution.

8.2.2 *Hybridisation*

(i) Except for single-stranded probes such as RNA and M13 probes, denature the labelled probe by placing it in a boiling water bath for 5 min. Quench in ice.

(ii) The hybridisation can be carried out in the presence of dextran sulphate which increases the rate of hybridisation (Section 3.8) or in its absence. For hybridisation buffer containing dextran sulphate (prepared as described in *Table 4*), pre-warm the buffer and add the denatured probe to a concentration which does not exceed 10 ng probe/ml or high backgrounds may ensue. In the absence of dextran sulphate, the probe concentration can be increased to 50−100 ng (43). For radioactive probes which have been labelled to a specific activity of

Table 4. Preparation of Standard Hybridisation Solution Containing Formamide.

Solution A	
Mix together:	
Deionised formamide[a]	50 ml
20 x SSC[b]	25 ml
100 x Denhardt's solution[c]	1 ml
1 M sodium phosphate buffer, pH 6.8[d]	2 ml
20% SDS[e]	1 ml
Dextran sulphate (mol. wt. 500 000)[h]	10 g
Stir until the dextran sulphate has dissolved. Adjust the volume to 95.5 ml.	
Solution B	
Mix together:	
Sonicated DNA (5 mg/ml)[f]	2 ml
Poly(C) [5 mg/ml][g]	0.2 ml
Poly(A) [5 mg/ml][g]	0.2 ml
Yeast tRNA (5 mg/ml)[g]	2 ml
Denature in a boiling water bath for 5 min.	
Quench in ice.	
Add solution B to solution A and store at 4°C.	

[a–g]See corresponding footnotes to *Table 3*.
[h]The inclusion of dextran sulphate is optional (see text, Section 3.8).

$1-2 \times 10^8$ c.p.m./μg, a probe concentration of 10 ng/ml gives a solution of about $1-2 \times 10^6$ c.p.m./ml).

(iii) Immediately add this solution to the filter (0.05 ml/cm^2 filter) and reseal the bag.

(iv) Hybridise the filter at 42°C for the required time. This is normally between 6 and 48 h. Overnight is convenient and for many purposes is sufficient, but see Section 11.2.

8.2.3 *Washing*

(i) Cut one corner from the bag and remove the hybridisation solution. Retain the probe if it is to be re-used (see Section 13), otherwise discard it down a designated sink.

(ii) Cut open the bag completely and immerse the filter in 200 ml of 2 x SSC, 0.1% SDS at room temperature. Shake the filter gently. Rinse the filter twice for 5 min each time in this solution.

(iii) For a moderately stringent wash, wash the filter twice in 400 ml of 2 x SSC. 0.1% SDS at 60°C for 1 h. For a higher stringency wash, treat the filter for 2 x 1 h at 65°C in 0.1 x SSC, 0.1% SDS.

(iv) Finally, rinse the filter in 2 x SSC at room temperature. Blot the filter to remove excess liquid but do *not* dry the filter if it is to be re-washed or re-screened (Section 13).

(v) Detect the hybrids as described in Section 10.1.

8.2.4 *Washing with Nuclease Treatment*

In principle, blots can be treated with nucleases to remove unpaired loops and single-

stranded probe tails. This practice should increase the specificity of the reaction, but it has not been studied systematically and there is no real evidence that the treatment is effective. It is better to control specificity by careful choice of reaction and dissociation conditions than through enzyme digestion. DNase and nuclease S1 treatments are not generally used in filter hybridisations, but RNase treatment occasionally is. Filters are treated with a mixture of RNase A and T1 RNase at 25 μg/ml and 10 units/ml, respectively, in 2 x SSC at 37°C for 2 h, then washed in 2 x SSC, 0.5% SDS at 68°C, and finally in 2 x SSC at room temperature.

8.3 Hybridisation in Aqueous Solution

8.3.1 *Pre-hybridisation*

(i) Wet the filter in 1% Triton X-100 and blot to remove excess liquid.

(ii) Immerse in 4 x SET buffer for 15 min at room temperature. The composition of 4 x SET buffer is 0.6 M NaCl, 1 mM EDTA, 80 mM Tris-HCl, pH 7.8.

(iii) Transfer the wet filter to a plastic bag and heat seal this except for one corner (see Section 8.2.1). Add pre-hybridisation buffer (0.08 ml/cm² of filter), prepared as described in *Table 5* and pre-warmed to 68°C. Incubate at 68°C for between 2 and 16 h.

(iv) Open the bag and remove the pre-hybridisation buffer (see Section 8.2.1 for methodology).

8.3.2 *Hybridisation*

(i) Denature the probe as described in Section 8.2.2 and add it to fresh pre-hybridisation buffer (pre-warmed to 68°C) at 10−25 ng/ml. Add this solution to the filter at 0.05 ml/ cm² of filter.

(ii) Incubate at 68°C for between 5 and 16 h.

Table 5. Preparation of Aqueous Pre-hybridisation Buffer.

Solution 1	
Mix together:	
20 x SET buffer[a]	20 ml
100 x Denhardt's solution[b]	10 ml
20% SDS[b]	0.5 ml
5% sodium pyrophosphate	0.1 ml
Adjust the volume to 97.5 ml with water.	
Solution 2	
Mix together:	
Sonicated DNA (5 mg/ml)[b]	2 ml
Poly(C) [5 mg/ml][b]	0.2 ml
Poly(A) [5 mg/ml][b]	0.2 ml
Denature in a boiling water bath for 5 min.	
Quench in ice.	
Add to solution 2 to solution 1 and store at 4°C.	

[a] 1 x SET = 0.15 M NaCl, 20 mM Tris-HCl, pH 7.8, 1 mM EDTA.
[b] See relevant footnote to *Table 3*.

8.3.3 *Washing*

(i) Cut one corner from the bag and remove the hybridisation solution. Retain the probe if it is to be re-used (see Section 13), otherwise discard it down a designated sink.

(ii) Cut open the bag completely. Remove the filter and immerse it in 4 x SET buffer, 0.1% SDS, 0.1% sodium pyrophosphate for 5 min at room temperature.

(iii) Wash the filter three times in 2 x SET, 0.1% SDS, 0.1% sodium pyrophosphate for 20 min each wash at 68°C.

(iv) For a moderately stringent wash, wash the filter three times in 1 x SET, 0.1% SDS, 0.1% sodium pyrophosphate for 20 min each wash at 68°C. For a higher stringency wash, replace the 2 x SET by 0.1 x SET.

(v) Rinse the filter for 5 min in 2 x SET, at room temperature.

(vi) Blot the filter to remove excess liquid but do *not* dry the filter if it is to be re-washed or re-screened (Section 13).

(vii) Detect the hybrids as described in Section 10.1.

9. HYBRIDISATION USING BIOTIN-LABELLED PROBES

The hybridisation procedure for biotin-labelled probes is essentially the same as for radioactively-labelled probes (Section 8) except that the following points should be noted (39,40):

(i) The probe should be denatured at high temperature and not with alkali because the amide bond in the linker molecule between the biotin and nucleic acid is alkali-labile.

(ii) Hybridisation is carried out in solutions containing formamide rather than at high temperature. The thermal stability of biotin-labelled hybrids is slightly lower than that of radioactive hybrids. So, in practice, the formamide concentration is lowered from 50% to 45% in otherwise standard hybridisation conditions.

(iii) Certain types of polythene bags are not suitable for hybridisation with biotin-labelled probes as they lead to high backgrounds. Layflat polythene tubing (Trans Atlantic Supplies) and Sears' Boilable Cooking Pouches are both suitable.

(iv) Since very low background signals are obtained with biotinylated probes, the concentration of probe can be increased to 250—750 ng/ml in the hybridisation solution. This has the additional advantage of allowing short hybridisation times of 1—2 h.

10. DETECTION AND QUANTITATION OF HYBRIDS

The detection of hybrids involving probes labelled with non-radioactive markers is described in Chapter 2, Section 4.3. Here we shall consider only radioactive hybrids.

10.1 Detection

For detecting [^{32}P]hybrids, autoradiography is the most commonly used technique. It is sensitive, gives good resolution and does not involve destruction of the filter.

If the filter is not to be re-screened or re-used, dry it at room temperature or under an infra-red lamp. Then expose it to X-ray film (e.g., Kodak X-Omat RP) at room

temperature in a light-proof cassette. The time of exposure will vary from several hours to 14 days depending on the level of radioactivity in the hybrids. As a rough guide, a dot containing 100 c.p.m. ^{32}P will give a good signal on X-ray film with an overnight exposure. If the radioactivity levels are low, use of an intensifying screen (e.g., Ilford fast tungstate or Fuji Mach II) increases the sensitivity of the film by 4- to 5-fold. The film is sandwiched between the filter and the intensifying screen in the cassette. Exposure is at −70°C because fluorescence reflected off the intensifying screen is prolonged at low temperatures. If two intensifying screens are used, the sensitivity of the film is enhanced 8—10 times. In this case the filter and film are sandwiched between the two intensifying screens. For low levels of radioactivity, the film can be pre-flashed and placed flashed side against the intensifying screen with the filter on top (Chapter 5, *Table 3*, step 15). As few as 5—10 c.p.m. per dot above background can be evaluated dependably by this adaptation of autoradiography.

If the filter is to be re-used (Section 13.1), it must not be allowed to dry otherwise the probe will bind irreversibly. The wet filter is covered in Clingfilm or Saranwrap or is inserted into a thin polythene sleeve before exposure to X-ray film. The filter should not be too wet or ice crystals will form when the cassette is placed at −70°C. This will distort the filter and could cause it to crack.

10.2 Quantitation of Hybridisation Signals

For many purposes it is sufficient to compare visually the intensity of hybridisation signal on an autoradiogram with that generated by a standard series of dots. The accuracy is better than 2-fold over a 100-fold range, taking into account both the intensity and diameter of the autoradiographic spots. For example, *Figure 4* shows dot blots of genomic DNA from patients suffering from chronic myeloid leukaemia (CML) and from two CML cell lines, K562 and NALM-1, probed with the c-*sis* and c-*abl* oncogenes and a human immunoglobulin λ light chain variable gene sequence (IgV_λ). Visual comparison of the autoradiographic signals indicates that cell line K562 contains about four times more copies of the c-*abl* and IgV_λ genes than the other cell lines.

For more accurate quantitation, densitometry can be used. This is a very simple and sensitive procedure; as little as 5—10 c.p.m. above background can be evaluated reliably. It is the best method of quantitation when the amount of radioactivity in hybrids is low. A scan is made of a series of standard dots and of the unknown samples. The area under the peaks is integrated, either electronically or the peaks can be cut from paper traces and weighed. The weight of the paper is a measure of the autoradiographic signal. A graph is then plotted of the weight of (or area under) the standard peaks against the known amount of nucleic acid on the filter. The concentration of the probe must be in excess over that on the filter and the autoradiograph should not be overexposed. An example of densitometric quantitation of a blot is given in *Figure 5*. Note that the curve relating intensity of signal (area under the peak) to the amount of RNA in the dot is only linear for a restricted range of amounts of filter-bound RNA. So, for the probe used in *Figure 5*, quantitation can be carried out only over the range 0—6 μg RNA per dot since beyond this the filter-bound sequences are in excess of this probe. The curve in *Figure 5c* does not reach a plateau in the range analysed because the size of the dots is not uniform.

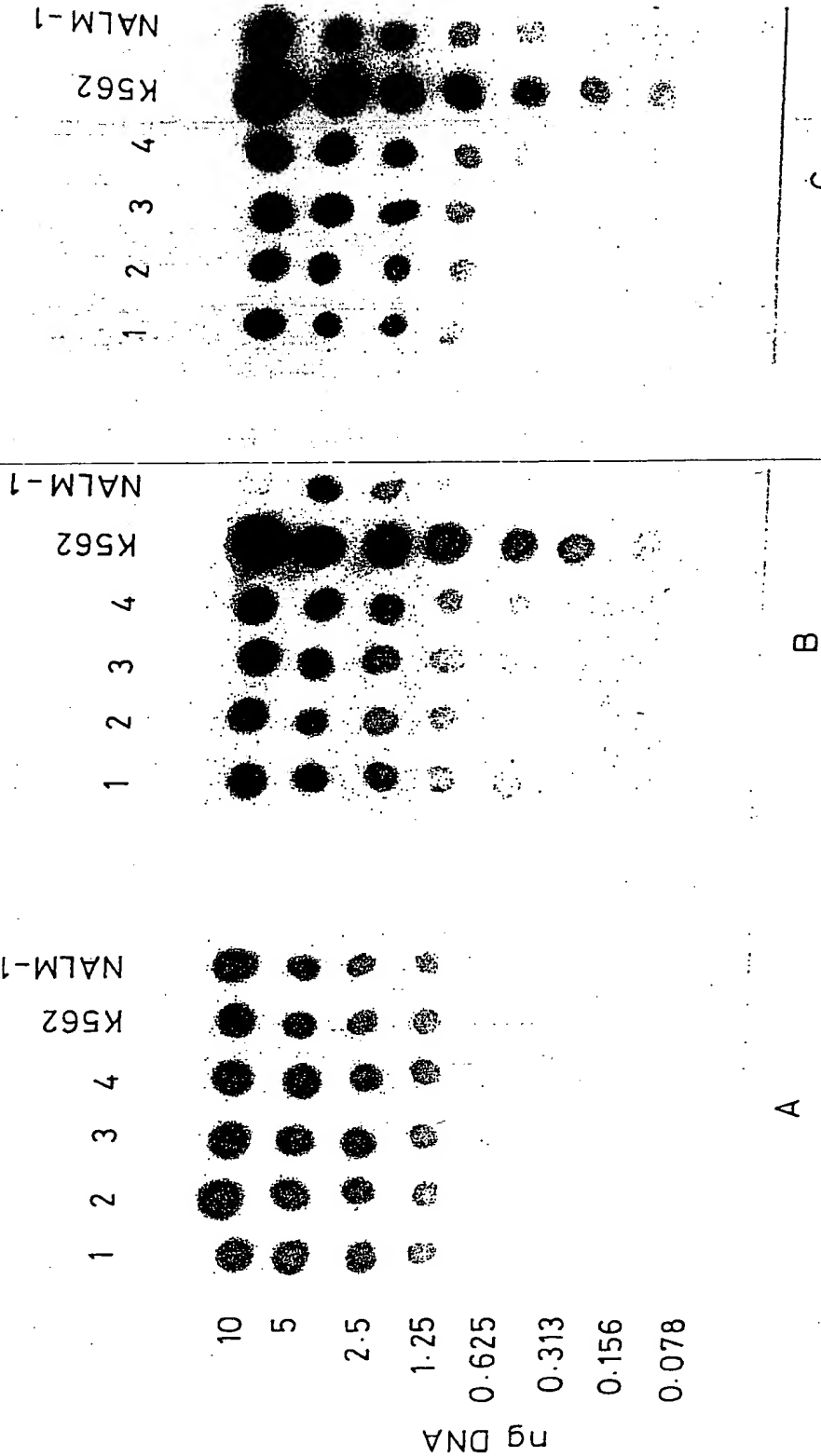

Figure 4. Genomic DNA dot blots. Replicate nitrocellulose filters containing the indicated amounts of genomic DNA from a dilution series were probed with (A) ^{32}P-labelled c-*sis* oncogene, (B) c-*abl* oncogene and (C) Ig V_λ DNA. Lanes 1–4 contained DNA from peripheral blood of chronic myeloid leukaemia (CML) patients, 5 and 6 contained DNA from CML cell lines K562 and NALM-1, respectively.

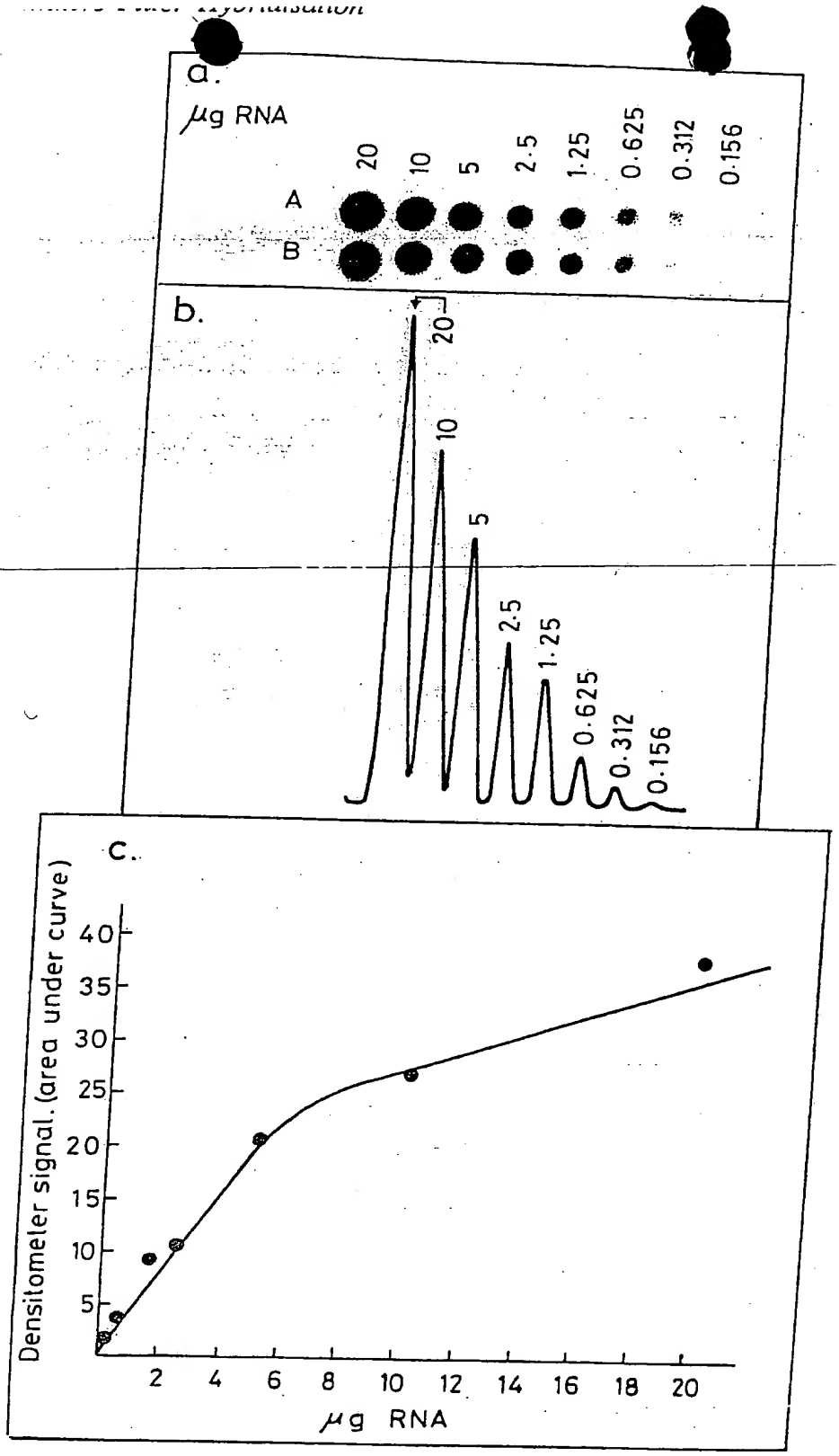

Figure 5. Quantitation of RNA dot blots. **(a)** Serial dilutions of RNA from patient 2 in *Figure 3* were prepared and applied in duplicate (A,B) to a nitrocellulose filter. The filter was hybridised with the Ha-*ras*-1 probe (*Figure 3*). **(b)** Densitometric scan made across lane A. **(c)** The relationship between the amount of RNA in each dot and the area under the densitometric peak for each dot.

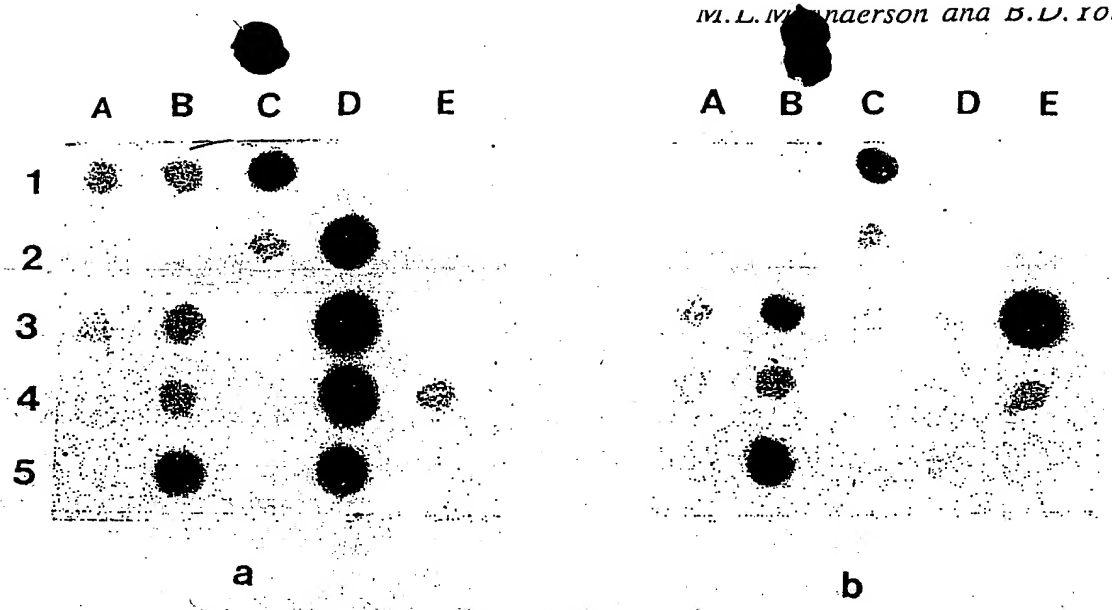

Figure 6. Plasmid DNA dot blots. Duplicate filters contained cloned recombinant plasmid cDNAs derived from mRNA of a patient suffering from acute non-lymphocytic leukaemia (ANLL). Probes were [^{31}P]cDNA derived from unfractionated mRNA of (a) ANLL and (b) chronic lymphocytic leukaemia (CLL) patients. The DNA bound to the filter was in significant excess over the probe. Data from M. Warnock, with permission.

If the hybrids are sufficiently radioactive, the dots can be cut out and counted in a liquid scintillation counter. This means, of course, that the filter cannot be re-hybridised.

10.3 Quantitative Analysis of Nucleic Acid Complexity

As we have seen, the rate of hybridisation is inversely proportional to the complexity of nucleic acid for both solution hybridisation and nucleation-limited filter hybridisation although not for diffusion-limited filter hybridisation (Section 2). Reassociation kinetics in solution have been used extensively to analyse the complexity of DNA and RNA populations and it might be supposed that nucleation-limited filter hybridisation could be used for a similar purpose. However, filter hybridisation is not suitable for quantitative studies of complexity. This is because the rate of filter hybridisation is so low that it is difficult to obtain C_0t values high enough for single copy sequences to hybridise (see Section 2).

10.4 Measurement of Relative Abundance of RNA Transcripts

For high and medium-abundance classes, dot blot hybridisation can be used to measure the relative prevalences of different mRNA species (44,45). Cloned recombinant cDNAs are applied in dots to filters and hybridised with either labelled mRNA or the cDNA derived from it. Filter-bound DNA is in excess so the extent of hybridisation is a measure of the concentration of the cloned cDNA sequence in the mRNA probe. [The extent of reaction is a reproducible characteristic of each clone and not a function of the cloned insert length, at least between the limits of 400 – 1500 nucleotides tested (44,45)]. It is estimated that a clone must be represented to a level of at least 0.1% of the mass of mRNA to be detected (44,46). This is probably true for optimal reaction conditions, but in practice the lower limit is more likely to be nearer 0.5%. An example is shown in *Figure 6*. Recombinant cDNA clones were constructed using mRNA from a patient

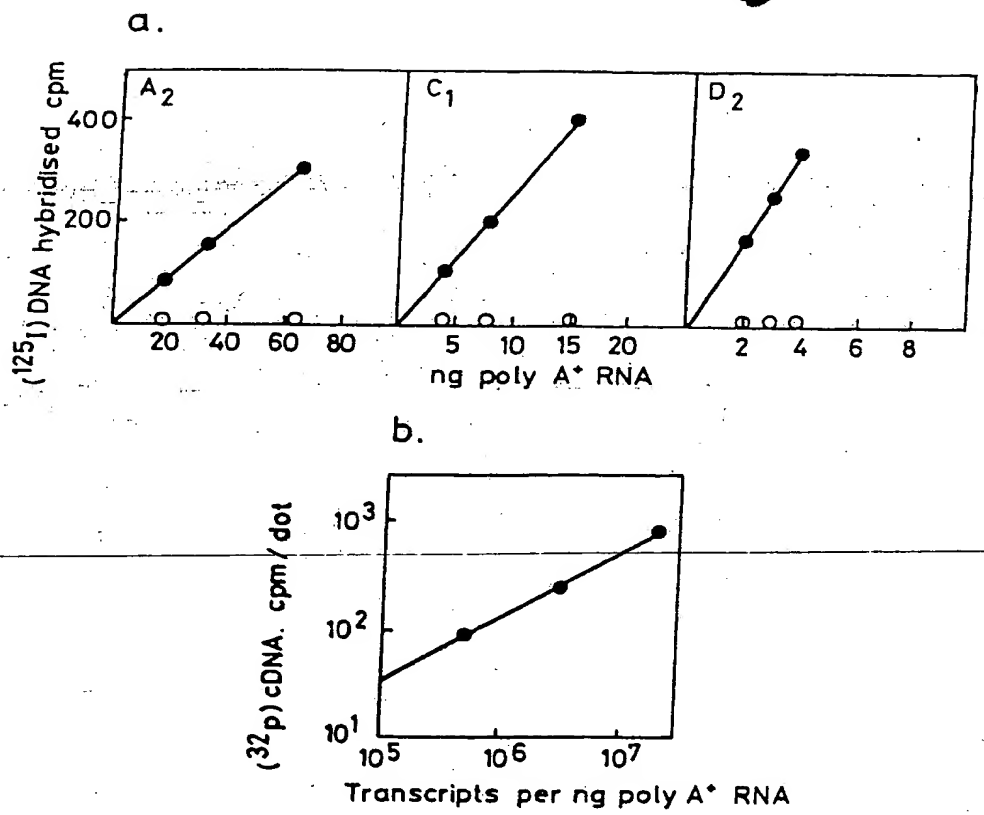

Figure 7. Prevalence analysis of mRNA transcripts. (a) Titration of cDNA clones with poly (A)$^+$ mRNA. Complementary strands (open and closed circles) of [^{125}I] cDNA recombinant plasmids A2, C1, and D2 (*Figure 6*) are hybridised separately in solution with the indicated amounts of poly(A)$^+$ RNA from ANLL cells. Reactions are carried out to 20 x $C_0t_{1/2}$ calculated with respect to the labelled DNA which is in sequence excess. The RNA-DNA hybrids formed in the reaction are analysed by resistance to nuclease S1. The slope of the lines was used to determine the number of transcripts per ng RNA. (Section 10.2).(b) Relationship between [^{32}P]cDNA dot blot hybridisation (*Figure 6*) and the number of transcripts per ng RNA for clones A2, C1 and D2.

suffering from acute non-lymphocytic leukaemia (ANLL). These were screened with [^{32}P]cDNA synthesised using unfractionated mRNA from an ANLL patient as template and with [^{32}P]cDNA complementary to unfractionated mRNA of a patient suffering from chronic lymphocytic leukaemia (CLL). Recombinant clones representing mRNAs common to the two diseases (e.g., B5, C1), and their relative abundance, can be easily identified and distinguished from those apparently specific to ANLL (e.g., D2, D3. D4, D5).

By using a calibration curve, the extent of hybridisation of [^{32}P]cDNA to each filter-bound recombinant can be used to determine the actual number of transcripts of mRNA (47,48). From the dot blot results (*Figure 6*), at least three cDNA clones whose representation in mRNA differs in abundance over a wide range are selected (e.g., clones A2. C1 and D2). Separated single strands of these recombinant DNAs are radiolabelled and hybridised separately in solution to different amounts of mRNA. The concentration of reactants is adjusted such that recombinant DNAs are in sequence excess. The reactions are carried out to about 20 x $C_0t_{1/2}$. The radioactivity in nuclease S1-resistant hybrids is determined and plotted against the amount of mRNA added to the reaction.

As expected, only one of the separated strands of DNA hybridises with the mRNA (*Figure 7a*). The number of transcripts per ng mRNA is then determined from the slope of the line using the relationship:

$$T = \frac{fN}{LS \times 350 \times 10^9}$$

where T is the number of transcripts per ng mRNA, f is the slope of the titration curve (c.p.m./ng mRNA), N is Avogadro's Number (number of molecules/ mol), L is the length of hybrid (i.e., cDNA insert length in nucleotides), S is the specific activity of the labelled cDNA (c.p.m./ng), and 350 is the average molecular weight of a ribonucleotide.

The appropriate values for clones A2, C1 and D2 of *Figure 7a* are:

Clone	*Specific activity* (S)	*Length of insert* (L)	*Slope* (f)	*No. of transcripts/ng mRNA* (T)
A2	2.0×10^4	800	4.7	5×10^5
C1	1.5×10^4	1000	26.3	3×10^6
D2	1.0×10^4	750	89.4	2×10^7

Using these values of T, a graph is plotted of the number of transcripts against the radioactivity in the hybrids on the dot blots. A linear relationship is obtained (*Figure 7b*). From this graph, the number of transcripts of any other recombinant on the dot blot matrix can easily be obtained.

11. DETERMINATION OF OPTIMAL REACTION CONDITIONS

11.1 Buffer Composition and Temperature

To determine the optimal reaction conditions, prepare replicate dot blots. Hybridise some under different hybridisation conditions, keeping the washing conditions constant, and monitor the effects. Then hybridise other dot blots under optimal hybridisation conditions and vary the washing conditions. For an extensive analysis of the effects of altering conditions on the hybrids formed, the reader is referred to references 3 – 5 and 43. The following is a rough guide.

(i) Reaction conditions which favour the detection of well-matched hybrids involve high temperatures of hybridisation (65 – 68°C in aqueous solution and 42°C in 50% formamide) combined with washing at high temperatures (5 – 25°C below T_m) and at low salt concentrations (0.1 x SSC).

(ii) To detect poorly-matched hybrids, filters should be hybridised in solutions containing formamide (20 – 50%) at 35 – 42°C but washed at high salt concentrations at an intermediate temperature (e.g., 2 – 6 x SSC at 40 – 60°C). Again, conditions may have to be determined empirically. It should be remembered that both closely-related and distantly-related sequences will be detected under these conditions.

(iii) To distinguish between closely- and distantly-related members of the same family, conditions must be found which are permissive for some sequences and stringent for others. As we have already seen (Section 5), distant homologies are best detected when the ratio of rate constants for hybridisation of cross-hybridising to self-hybridising species is high, whereas closely-related species are most easily detected when the ratio is low. In practical terms, this means that for distantly-related hybrids low temperatures of incubation are used, whereas for closely-related hybrids high temperatures are best. The time of incubation is very important since the effective discrimination between closely- and distantly-related hybrids is highest with very short times of incubation and deteriorates very rapidly thereafter (Section 5). If short incubations do not give a sufficiently high hybridisation signal, then longer times can be used with excess filter-bound nucleic acid.

11.2 Time Period of Incubation

It is difficult to give a precise time period for hybridising filters. Filter hybridisations tend not to go to completion. As described above, the rate of hybridisation on filters is about 10 times slower than that for solution hybridisation of the same DNAs (9), so it is difficult experimentally to reach the very high C_0t values required for complete hybridisation. Prolonged incubation does not necessarily increase the extent of hybridisation because:

(i) more and more probe reassociates
(ii) at the high temperatures involved, sequences leach off the filter if they are not covalently bound
(iii) the probe is gradually degraded.

Addition of formamide to the hybridisation solution allows lower temperatures to be used and thus incubation times can be extended, but there is still the problem of probe reassociation unless a single-stranded probe is used. Furthermore, steric constraints prevent all the bound sequences being nucleated effectively. Under optimal conditions, no more than 80% of a single sequence probe appears in hybrids (9) and so an even smaller proportion of more complex probes will be hybridised.

In practice, for double-stranded DNA probes, there is no need to proceed for longer than to allow the probe in solution to achieve $1-3 \times C_0t_{1/2}$. After incubation for $3 \times C_0t_{1/2}$, the amount of probe available for additional hybridisation to sequences on the filter is negligible. The following useful guideline is taken from ref. 46. In 10 ml hybridisation solution, 1 μg denatured double-stranded probe with a complexity of 5 kb will reach $C_0t_{1/2}$ in 2 h. To determine the number of hours (n) needed to achieve $C_0t_{1/2}$ for renaturation of any other probe, the appropriate values can be substituted in the following equation:

$$n = \frac{1}{X} \times \frac{Y}{5} \times \frac{Z}{10} \times 2$$

where X is the weight of the probe added (in μg), Y is its complexity (which for most probes is proportional to the length of the probe in kb) and Z is the volume of the

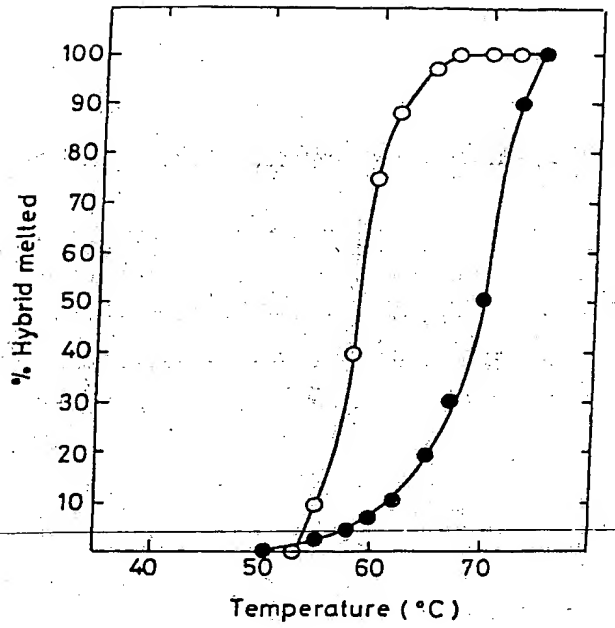

Figure 8. Melting profile of dot hybrids. Replicate filters containing dots of linearised DNA are hybridised to homologous ^{32}P-labelled DNA (○) or RNA (●). Hybridisation, in a formamide-containing solution. is carried out to 20 x $C_0t_{1/2}$. The DNA bound to the filter is present in significant excess. After hybridisation each filter is washed in the same formamide-containing buffer as used for hybridisation, with stepwise increases in temperature. The filters are counted by Cerenkov counting between each washing step to determine the percentage of hybridised [^{32}P]DNA or [^{32}P]RNA which has eluted (melted) at each temperature.

reaction (in ml).

For quantitative dot blots (e.g., *Figure 6*) the time period of incubation, the concentration of probe and the amount of nucleic acid bound to the filter should all be adjusted such that there is low fractional hybridisation of both the probe and filter-bound sequences. This is to ensure that the proportion of the probe hybridising increases linearly with time.

Factors affecting the selection of a time of incubation suitable for discrimination between related sequences are discussed in Section 5.2 [see also Section 11.1 (iii)].

12. MEASUREMENT OF T_m

The DNA or RNA sample is applied in a dot to a filter and hybridised with a labelled DNA or RNA probe as appropriate. It is then washed at the same temperature in a solution of the same composition as was used for the hybridisation. This removes unhybridised and non-specifically-bound probe. For melting of the hybrid, the filter is incubated for 10 – 15 min in a small volume of the same buffer at progressively higher temperatures. The buffers used must be pre-heated to the required temperature. Between melting steps, the filter is counted by Cerenkov counting, making sure that it does not dry out at any stage in order to prevent irreversible binding of the probe. Replicate filters can be hybridised at different criteria or washed in solutions of different ionic strength. *Figure 8* shows the melting temperature profiles to be expected from homologous DNA-DNA and RNA-DNA hybridisations. Note that in formamide-containing

solutions RNA-DNA hybrids are more stable than the corresponding DNA-DNA hybrids and so dissociate (melt) at higher temperatures. The exact temperature difference will depend on the base composition of the nucleic acids involved, the size of the probe and the degree of relatedness of the two hybridising species. In the example given in *Figure 8*, the difference in T_m is 11°C.

For a single nucleic acid species hybridising to itself, the hybrid melts over a very narrow temperature range and the T_m is the same irrespective of the incubation temperature, T_i. However, when the same nucleic acid is probed with a complex mixture of sequences which have varying degrees of relatedness, the T_m profile depends on the reaction conditions. For hybrids formed at low criterion (T_m −25°C), the melting profile is broad because both well-matched and poorly-matched hybrids are formed. They melt at different temperatures, so the overall melting profile, which is a composite of the contributions of all the hybrids, will reflect this. At high criterion (T_m −8°C), only hybrids with a high degree of homology form so they melt over a very narrow temperature range. The melting profile is also broad when variable length probes are used. This is most apparent at short average lengths of hybridised probe in accordance with the empirical relationship:

$$T_n - T_m = \frac{650}{L}$$

where L is the length of the probe in nucleotides, T_m is the melting temperature of the short hybrid and T_n is the melting temperature of long DNA molecules (17).

The procedure of stepwise melting of hybrids described above for T_m measurement can be extended to investigate the degree of relatedness of different sequences and can be applied to many samples at once. An array of dots on a single filter are hybridised to a labelled probe and the extent of similarity to the probe is evaluated by stepwise melting and autoradiography (3). The more mismatched a hybrid is, the lower the temperature at which it will melt. Thus differences in the intensity of signal of the dots can be interpreted in terms of the degree of relatedness of the different sequences.

13. RE-USE OF FILTERS AND PROBES

13.1 Filters

In many cases, re-probing the same filters with a series of different probes yields valuable information. Filters can be re-probed several times — the exact number being dependent on the type of filter and the incubation conditions to which the filter has already been exposed. Nitrocellulose filters which have been exposed to high temperatures for hybridisation and washing can be used about two to four times before falling apart. Filters exposed to the less harsh conditions of hybridisation at lower temprature in the presence of formamide can be used many more times. Nylon filters can be used indefinitely without disintegration and so, because of their superior durability, they are preferred to nitrocellulose for multiple probings.

Before re-probing a filter with a new probe, it is first necessary to strip off the old probe and to monitor that the treatment has been effective. This can be achieved for both DNA and RNA dot blots as follows:

(i) Transfer the damp filter to a plastic box containing 200 ml of 5 mM Tris-HCl. pH 8.0, 0.1 mM EDTA, 0.05% sodium pyrophosphate, 0.1% Denhardt's solution (see *Table 3*, footnote c for composition) at 65°C. Incubate for 1 h with gentle agitation.

(ii) Discard the wash buffer and repeat step (i).

(iii) Check by autoradiography as described in Section 10.1 that the probe has been removed.

An alternative procedure for DNA dot blots only is given below.

(i) Wash the filter twice, for 10 min each wash, in 50 mM NaOH at room temperature.

(ii) Wash and neutralise the filter by incubating it (5 min each time) in five changes of TE buffer, pH 7.5 (see *Table 1*, footnote a) at room temperature.

This procedure cannot be carried out successfully for RNA dot blots since the NaOH hydrolyses the filter-bound RNA.

Unfortunately there are several potential problems with re-using filters:

(i) Loss of sensitivity. Prolonged use of both nylon and nitrocellulose filters leads to gradual reduction in sensitivity through loss of filter-bound nucleic acid (Section 6.1). This is not a problem if the nucleic acid has been covalently bound to the filter matrix.

(ii) Irreversible binding of the previous probe if the filter was allowed to dry.

(iii) Incomplete removal of the previous probe even if the filter was kept wet throughout its use. This can give misleading results if single-stranded tails of probe remaining on the filter are complementary to sequences of the new probe. For example, if the first probe is a recombinant DNA (vector and insert) and the second is also a recombinant DNA with a different insert, hybridisation may occur through plasmid sequences and this will obscure the hybridisation of the second probe insert sequences. Therefore, it is recommended that all inserts are excised from vectors before labelling as probes.

13.2 Probes

Normally only a small fraction of the probe is used up during hybridisation, so probes can be re-used until they are degraded or have decayed to too low a specific activity. To re-use the probe (now in the hybridisation solution) it must be denatured again by heating to a temperature above its T_m. For aqueous solutions, this can be done by incubating in a boiling water bath for 10 min. For formamide-containing solutions, heat at 70°C for 30 min. The newly-denatured probe can now be added to a second filter which has been pre-hybridised under standard conditions.

14. PROBLEMS

Most hybridisation experiments using filter-bound nucleic acids employ radioactive probes and so problems of only this type of investigation are covered here.

(i) The autoradiograph of the filter is black all over:

(a) At some stage during hybridisation or washing, the filter was allowed to

dry. It will probably be necessary to strip the filter (Section 13.1) and re-hybridise.

(b) The probe is 'dirty'. It may be contaminated by traces of agarose. Either re-purify the nucleic acid from which the labelled probe was derived and prepare a new probe, or pass the labelled probe through a nitrocellulose filter which has been pre-treated in 10 x Denhardt's solution or through a mini NACS column (BRL).

(c) An inappropriately low hybridisation and/or washing temperature was used.

(ii) The autoradiograph of the filter is black in parts:

(a) Part of the filter dried out; see above.

(b) The filter was handled with bare hands. Grease marks from fingers trap probe. Wear disposable plastic gloves in future.

(iii) The autoradiograph has black dots in random locations:

(a) The unincorporated precursors were not completely removed from the probe. See correct procedure in relevant section of Chapter 2.

(b) Air bubbles were not completely removed from the bag during hybridisation. (This may not matter if a shaking water bath is used, but the effect may be quite troublesome if the bag is not agitated.)

(c) Dust or dirt on the filter. Filter all solutions before use in future.

(iv) The signal is lower than expected:

(a) Was the correct binding procedure used? Nitrocellulose and nylon filters use different binding protocols (see Section 6.1).

(b) The probe was degraded. This is most likely to happen with RNA probes.

(c) The double-stranded probe was not denatured (see Section 8.2.2).

(d) The hybridisation and/or washing conditions were too stringent so that the hybrids either did not form at all, or were dissociated.

(e) The specific activity of the probe was too low.

(f) The hybridisation time was too short.

(g) The filter was not exposed to film for long enough.

(v) A 'negative' effect is obtained, that is, the background of the autoradiograph is black with clear dots. Too high a concentration of [^{32}P]probe was used.

(v) The filter fell apart. This is most likely to occur with nitrocellulose filters.

(a) During binding of DNA to the filter, the alkali was not properly neutralised thus making the filter yellowish in colour and very brittle.

(b) After repeated use, the filter becomes brittle despite correct procedures. Prepare new filters.

15. ACKNOWLEDGEMENTS

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[19] **United States Patent**

Dower et al.

[11] **Patent Number: [redacted],968,607**

[45] **Date of Patent: Nov. 6, 1990**

[54] **INTERLEUKIN-1 RECEPTORS**

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[21] Appl. No.: **160,550**

[22] Filed: **Feb. 25, 1988**

Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 125,627, Nov. 25, 1987.

[51] Int. Cl.[5] C12N 15/00; C12N 15/12; C12N 12/11; C12N 0/6

[52] U.S. Cl. 435/69.1; 536/27; 435/320; 435/235; 435/240.1; 435/252.8; 435/255; 330/387; 330/399

[58] Field of Search 536/27; 435/68, 172.3, 435/235, 320, 240.1, 69.1, 252.8, 255; 530/387, 399

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Primary Examiner—Robin L. Teskin
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[57] **ABSTRACT**

Mammalian Interleukin-1 receptor proteins (IL-1Rs), DNAs and expression vectors encoding mammalian IL-1Rs, and processes for producing mammalian IL-1Rs as products of cell culture, including recombinant systems, are disclosed.

21 Claims, 14 Drawing Sheets

cDNA set forth in FIG. 2. The initiator methionine for the full-length translation product of the native murine gene is one of two alternative methionine residues found at positions −19 and −16 of FIG. 3A. The first amino acid residue of the mature receptor protein was deduced by comparison to an N-terminal amino acid sequence obtained from highly purified preparations of IL-1R derived from EL-4 6.1 C10 cells. This residue is a leucine residue shown at position 1 of FIG. 3A. The 1671 nucleotide coding region corresponding to the mature protein encodes 576 amino acids, including 15 cysteine residues and a 21-amino acid putative transmembrane region. Located N-terminal to the transmembrane region are 7 potential N-glycosylation sites. A cloning vector comprising the full-length murine cDNA, designated GEMBL78, has been deposited with the American Typr Culture Collection, Rockville, MD, USA (ATCC) under accession number 67563. The deposit was made under the conditions of the Budapest Treaty.

A probe was constructed from the murine sequence and used to screen human cDNA libraries prepared from cultures of a human T-cell clone grown in the presence of OKT3 antibody and IL-2. cDNA clones which hybridized to the murine probe were then isolated and sequenced. Using a fragment derived from human cDNA clones, a 1707 nucleotide human coding sequence was obtained and sequenced. The nucleotide sequence of the human cDNA, including 5' and 3' nontranslated sequences, is shown in FIG. 4. The nucleotide sequence of the human open reading frame and derived amino acid sequence of the human protein is set forth in FIGS. 5A–5C. This sequence comprises 569 amino acids (including a 17 amino acid signal peptide), including 16 cysteine residues, 13 of which are conserved between the murine and human genes. In addition, the human sequence includes six potential N-glycosylation sites, of which 5 are conserved between murine and human. The amino acid sequence of FIGS. 5A–5C is numbered from a leucine residue considered to be the likely N-terminus on the basis of comparison to the murine protein. The putative transmembrane region of the human gene is 20 amino acids in length. The sequences of the presumed intracellular portions of the murine and human genes are highly (87%) conserved; the extracellular (78%) and transmembrane regions (63%) are somewhat less conserved, except for the location of cysteines presumably involved in intramolecular disulfide bonding and certain N-glycosylation sites. The derived amino acid sequences of the human and murine genes are compared in FIG. 8.

The murine and human genes encode integral membrane proteins including intracellular regions having no apparent homology with any known protein sequence and extracellular portions which appear to be organized into domains similar to those of members of the immunoglobulin gene superfamily. Immunoglobulin-like domains typically possess only minimal amino acid similarity but share a common three-dimensional structure consisting of two α-sheets held together by a disulfide bond. The cysteine residues involved in formation of this disulfide bond, as well as a few other critical residues, are highly conserved and occur in the same relative position in almost all members of the family. Members of the immunoglobulin superfamily include not only immunoglobulin constant and variable regions but also a number of other cell surface molecules, many of which are involved in cell-cell interactions.

Like most mammalian genes, mammalian IL-1Rs are presumably encoded by multi-exon genes. Alternative mRNA constructs which can be attributed to different mRNA splicing events following transcription, and which share large regions of identity or similarity with the cDNAs claimed herein, are considered to be within the scope of the present invention.

In its nucleic acid embodiments, the present invention provides DNA sequences encoding mammalian IL-1Rs. Examples of mammalian IL-1Rs include primate IL-1R, human IL-1R, murine, canine, feline, bovine, ovine, equine and porcine IL-1Rs. IL-1R DNAs are preferably provided in a form which is capable of being expressed in a recombinant transcriptional unit under the control of mammalian, microbial, or viral transcriptional or translational control elements. For example, a sequence to be expressed in a microorganism will contain no introns. In preferred aspects, the DNA sequence comprise at least one, but optionally more than one sequence component derived from a cDNA sequence or copy thereof. Such sequences may be linked or flanked by DNA sequence prepared by assembly of synthetic oligonucleotides. However, synthetic genes assembled exclusively from oligonucleotides could be constructed using the sequence information provided herein. Exemplary sequences include those substantially identical to the nucleotide sequences depicted in FIGS. 3A–3C. Alternatively, the coding sequences may include codons encoding one or more additional amino acids located at the N-terminus, for example, an N-terminal ATG codon specifying methionine linked in reading frame with the nucleotide sequence. Due to code degeneracy, there can be considerable variation in nucleotide sequences encoding the same amino acid sequence; exemplary DNA embodiments are those corresponding to the sequence of nucleotides 1–1671 of FIGS. 3A–3C, and nucleotides 1–1656 of FIGS. 5A–5C. Other embodiments include sequences capable of hybridizing to the sequence of FIGS. 3A–3C or 5A–5C under moderately stringent conditions (50° C., 2×SSC) and other sequences degenerate to those described above which encode biologically active IL-1R polypeptides.

The present invention also provides expression vectors for producing useful quantities of purified IL-1R. The vectors can comprise synthetic or cDNA-derived DNA fragments encoding mammalian IL-1Rs or bioequivalent homologues operably linked to regulatory elements derived from mammalian, bacterial, yeast, bacteriophage, or viral genes. Useful regulatory elements are described in greater detail below. Following transformation, transfection or infection of appropriate cell lines, such vectors can be induced to express recombinant protein.

Mammalian IL-1Rs can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems could also be employed to produce mammalian IL-1R using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are described by Pouwels et al. (*Cloning Vectors: A Laboratory Manual*, Elsevier, New York, 1985), the relevant disclosure of which is hereby incorporated by reference.

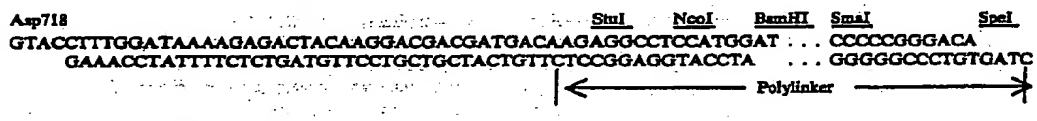
Various mammalian cell culture systems can be employed to express recombinant protein. Examples of suitable mammalian host cell lines include the COS-7 lines of monkey kidney cells, described by Gluzman

precipitation followed by gel exclusion chromatography, and/or affinity chromatography based on binding of antibody to Protein A of *Staphylococcus aureus*.

Example 8

Expression of IL-1R in Yeast

For expression of human or murine IL-1R in yeast, a yeast expression vector derived from pIXY120 is constructed as follows. pIXY120 is identical to pYαHuGM (ATCC 53157), except that it contains no cDNA insert and includes a polylinker/multiple cloning site with an NcoI site. This vector includes DNA sequences from the following sources: (1) a large SphI (nucleotide 562) to EcoRI (nucleotide 4361) fragment excised from plasmid pBR322 (ATCC 37017), including the origin of replication and the ampicillin resistance marker for selection in *E. coli*; (2) *S. cerevisiae* DNA including the TRP-1 marker, 2μ origin of replication, ADH2 promoter; and (3) DNA encoding an 85 amino acid signal peptide derived from the gene encoding the secreted peptide α-factor (See Kurjan et al., U.S. Pat. No. 4,546,082). An Asp718 restriction site was introduced at position 237 in the α-factor signal peptide to facilitate fusion to heterologous genes. This was achieved by changing the thymidine residue at nucleotide 241 to a cytosine residue by oligonucleotide-directed in vitro mutagenesis as described by Craik, *Biotechniques*:12 (1985). A synthetic oligonucleotide containing multiple cloning sites and having the following sequence was inserted from the Asp718 site at amino acid 79 near the 3' end of the α-factor signal peptide to a SpeI site in the 2μ sequence:



pBC120 also varies from pYαHuGM by the presence of a 514 bp DNA fragment derived from the single-stranded phage f1 containing the origin of replication and intergenic region, which has been inserted at the Nru1 site in the pBR322 sequence. The presence of an f1 origin of replication permits generation of single-stranded DNA copies of the vector when transformed into appropriate strains of *E. coli* and superinfected with bacteriophage f1, which facilitates DNA sequencing of the vector and provides a basis for in vitro mutagenesis. To insert a cDNA, pIXY120 is digested with Asp718 which cleaves near the 3' end of the α-factor leader peptide (nucleotide 237) and, for example, NcoI which cleaves in the polylinker. The large vector fragment is then purified and ligated to a DNA fragment encoding the protein to be expressed.

To create a secretion vector for expressing human IL-1R, a cDNA fragment including the complete open reading frame encoding hIL-1R is cleaved with an appropriate restriction endonuclease proximal to the N-terminus of the mature protein. An oligonucleotide or oligonucleotides are then synthesized which are capable of ligation to the 5' and 3' ends of the hIL-1R fragment, regenerating any codons deleted in isolating the fragment, and also providing cohesive termini for ligation to pIXY120 to provide a coding sequence located in frame with respect to an intact α-factor leader sequence.

The resulting expression vectors are then purified and employed to transform a diploid yeast strain of *S. cerevisiae* (XV2181) by standard techniques, such as those disclosed in EPA 0165654, selecting for tryptophan prototrophs. The resulting transformants are cultured for expression of an hIL-1R protein as a secreted or extracted product. Cultures to be assayed for hIL-1R expression are grown in 20–50 ml of YPD medium (1% yeast extract, 2% peptone, 1% glucose) at 37° C. to a cell density of 1–5×10⁸ cells/ml. To separate cells from medium, cells are removed by centrifugation and the medium filtered through a 0.45μ cellulose acetate filter prior to assay. Supernatants produced by the transformed yeast strain, or extracts prepared from disrupted yeast cells, are assayed for the presence of hIL-1R using binding assays as described above.

We claim:

1. An isolated DNA sequence encoding a mammalian IL-1 receptor (IL-1R protein, or soluble truncated IL-1R protein which exhibits IL-1-binding activity.

2. A DNA sequence according to claim 1 which encodes an IL-1R protein exhibiting IL-1-binding activity, selected from the group consisting of:

(a) cDNA clones having a nucleotide sequence derived from the coding region of a native mammalian IL-1R gene and which encode IL-1R protein;

(b) DNA sequences capable of hybridization to the clones of (a) under moderately stringent conditions and which encode biologically active IL-1R protein; and

(c) DNA sequences which are degenerate as a result of the genetic code to the DNA sequences defined in (a) and (b) and which encode biologically active IL-1R protein.

3. A recombinant expression vector comprising a DNA sequence according to claim 2.

4. A DNA sequence according to claim 1 which encodes an IL-1R protein exhibiting IL-1-binding activity, consisting essentially of a synthetic gene which encodes a mammalian IL-1R protein which is capable of being expressed in a recombinant transcriptional unit comprising inducible regulatory elements derived from a microbial or viral operon.

5. A recombinant expression vector comprising a DNA sequence according to claim 4.

6. A DNA sequence according to claim 1 which encodes an IL-1R protein exhibiting IL-1-binding activity, said protein having an amino acid sequence corresponding to the sequence of amino acids 1–557 depicted in FIGS. 3A–3C or a portion thereof which encodes a soluble truncated IL-1R protein which exhibits IL-1 binding activity.

7. A recombinant expression vector comprising a DNA sequence according to claim 6.

8. A process for preparing a murine IL-1R, comprising culturing a suitable host cell comprising a vector according to claim 14 under conditions promoting expression.

9. A DNA sequence according to claim 1 which encodes an IL-1R protein exhibiting IL-1-binding activity, said protein having an amino acid sequence corresponding to the sequence of amino acids 1–552 depicted

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United States Patent [19]

Cerretti et al.

[11] Patent Number: 5,171,675

[45] Date of Patent: Dec. 15, 1992

[54] MACROPHAGE COLONY STIMULATING FACTOR-γ

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[21] Appl. No.: 391,218

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[52] U.S. Cl. 435/69.5; 435/320.1; 530/399; 536/27

[58] Field of Search 435/69.5, 320.1; 536/27

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Primary Examiner—Richard A. Schwartz
Assistant Examiner—James S. Ketter
Attorney, Agent, or Firm—Christopher L. Wight

[57] ABSTRACT

A macrophage colony stimulating factor, M-CSFγ, which is a primary translation product of an alternative mRNA splicing event and is a precursor to biologically active M-CSF. DNA sequences encoding M-CSFγ and recombinant expression vectors comprising the DNA sequences.

18 Claims, 6 Drawing Sheets

subsequently cleaved from the expressed recombinant protein to provide a final product.

"Recombinant microbial expression system" means a substantially homogeneous monoculture of suitable host microorganisms, for example, bacteria such as *E. coli* or yeast such as *S. cerevisiae*, which have stably integrated a recombinant transcriptional unit into chromosomal DNA or carry the recombinant transcriptional unit as a component of a resident plasmid. Generally, cells constituting the system are the progeny of a single ancestral transformant. Recombinant expression systems as defined herein will express heterologous protein upon induction of the regulatory elements linked to the DNA sequence or synthetic gene to be expressed.

The synthesis of human M-CSF is more complex than the other hematopoietic growth factors. The gene for M-CSF transcribes multiple RNA species that range in size from 1.5 to 4.4 kb. After translation, M-CSF polypeptides are glycosylated, react with each other to form dimers and are inserted into the plasma membrane. The membrane-bound precursor is then cleaved, releasing secreted M-CSF. The two known cDNAs that encode biologically active M-CSF, namely M-CSFα and M-CSFβ, encode proteins of 256 and 554 amino acids, respectively. M-CSFγ of the present invention encodes a protein of 438 amino acids. While not being bound to any particular theory, it is believed that the cDNAs are likely a result of alternative RNA splicing within the coding region of the gene as judged by the presence of consensus splice sequences at the divergence points between the cDNAs. It is possible that the M-CSFβ mRNA could be spliced to give either the M-CSFα or M-CSFγ mRNAs by using alternative donor sites spliced to the same acceptor site. Alternatively, all three mRNAs could be produced by alternative splicing of a common precursor. The proteins encoded by M-CSFα, M-CSFβ and M-CSFγ have a common amino terminus of 149 amino acids including a 32 amino acid signal sequence and a common 75 amino acid carboxyl-terminus including a membrane-spanning region. Each of the M-CSF molecules is represented by a form anchored to the plasma membrane. M-CSFβ and M-CSFγ have an additional 298 and 182 amino acids, respectively, as compared to M-CSFα. This insertion is upstream and adjacent to the membrane spanning region. It is within this region that the membrane-bound precursors are likely to be proteolytically processed.

Isolation of cDNAs Encoding M-CSFγ

In order to identify the coding sequence of human M-CSFγ, a DNA sequence encoding human M-CSFγ was isolated from a cDNA library prepared by reverse transcription of polyadenylated mRNA isolated from mitogen-stimulated human pancreatic tumor cells, MIA-PaCa-2, which are known to produce significant levels of human M-CSF. The library was probed with an M-CSF probe ([s]M-CSFα) consisting of nucleotides 97 to 544 and 1439 to 1467 (FIG. 2). This fragment was assembled from 16 synthetic oligonucleotides and contains the first 158 amino acids of M-CSFα. Restriction mapping and DNA sequence analysis of eight hybridizing clones revealed three classes of M-CSF cDNAs (FIGS. 1A and 2). Two of the classes represent the M-CSFα (three isolates) and M-CSFβ (four isolates) cDNAs isolated previously by Kawaski et al. and Wong et al. However, a new class of M-CSF cDNA, referred to here as M-CSFγ (one isolate), was found that encodes a primary translation product intermediate in size to M-CSFα and M-CSFβ.

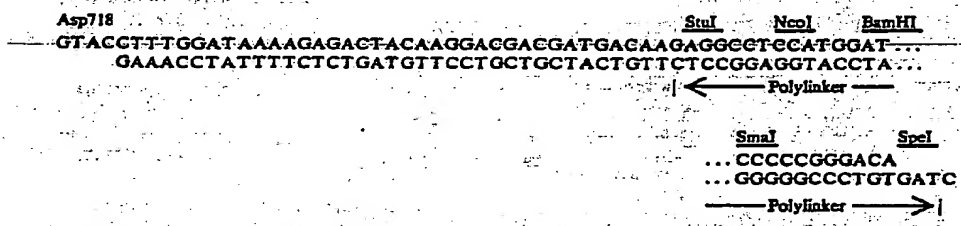
The M-CSFγ cDNA encoded a protein of 438 amino acids, 182 amino acids larger than encoded by M-CSFα and 116 amino acids smaller than encoded by M-CSFβ. M-CSFγ appears to be a result of an in-frame insertion of 546 bp (FIG. 2, #2 arrows) into M-CSFα at the same location as the 894 bp insertion forming M-CSFβ (FIG. 2, #1 arrows). Analysis of the DNA sequence (FIG. 2) shows that these 546 bp are identical to those found in M-CSFβ, indicating that the three cDNAs are probably a result of alternative splicing of M-CSF mRNA. The nucleotide sequences surrounding the insertions are similar to the consensus sequences found for mRNA donor, $^{C}_{A}G/GT^{A}_{G}GT$ and acceptor, $(^{T}_{C})_{n}N^{T}_{C}AG/G$ splice sites, as described by Mount (Nucl. Acids Res. 10:457, 1982). Splicing of the mRNA for M-CSFβ between the #1 and #2 arrows would form M-CSFα and M-CSFγ, respectively.

In its nucleic acid embodiments, the present invention provides DNA sequences comprising a single open reading frame nucleotide sequence encoding human M-CSFγ. M-CSFγ DNAs are preferably provided in a form which is capable of being expressed in a recombinant transcriptional unit under the control of mammalian, microbial, or viral transcriptional or translational control elements. For example, a sequence to be expressed in a microorganism will contain no introns. In preferred aspects, the DNA sequences comprise at least one, but optionally more than one sequence component derived from a cDNA sequence or copy thereof. Such sequences may be linked or flanked by DNA sequences prepared by assembly of synthetic oligonucleotides. However, synthetic genes assembled exclusively from oligonucleotides could be constructed using the sequence information provided herein. Exemplary sequences include those substantially identical to the nucleotide sequences depicted in FIG. 2. Alternatively, the coding sequences may include codons encoding one or more additional amino acids located at the N-terminus, for example, an N-terminal ATG codon specifying methionine linked in reading frame with the nucleotide sequence. Due to code degeneracy, there can be considerable variation in nucleotide sequences encoding the same amino acid sequence; exemplary DNA embodiments are those corresponding to the sequence of nucleotides in FIG. 2. Other embodiments include sequences capable of hybridizing to the sequence of FIG. 2 under moderately stringent conditions (50° C., 2X ssc) and other sequences degenerate to those described above which encode M-CSFγ.

Recombinant Expression of M-CSF cDNAs

In order to compare the molecular weight and biological activity of the protein encoded by M-CSFγ cDNA to those encoded by M-CSFα and M-CSF cDNAs, the coding regions for M-CSFα, M-CSFβ and M-CSFγ, were inserted into the mammalian expression vector, pDC201. The resulting plasmids, designated pDCCSFα, pDCCSFβ, pDCCSFγ, were transfected into COS-7 monkey kidney cells. After 72 hr, the cultures were labeled for 24 hr with ^{35}S-Met and ^{35}S-Cys and M-CSF specific proteins immunoprecipitated from the supernatants with a rabbit anti-M-CSF polyclonal antiserum. Immunoprecipitates were then subjected to SDS-PAGE under reducing conditions and protein bands visualized by autoradiography. The proteins synthesized by M-CSFβ and M-CSFγ cDNAs had a mo-

No. 4,546,082). An Asp718 restriction site was introduced at position 237 in the α-factor signal peptide to facilitate fusion to heterologous genes. This was achieved by changing the thymidine residue at nucleotide 241 to a cytosine residue by oligonucleotide-directed in vitro mutagenesis as described by Craik, Biotechniques: 12 (1985). A synthetic oligonucleotide containing multiple cloning sites and having the following sequence was inserted from the Asp718 site at amino acid 79 near the 3' end of the α-factor signal peptide to a SpeI site in the 2μ sequence:



pBC120 also varies from pYαHuGM by the presence of a 514 bp DNA fragment derived from the single-stranded phage f1 containing the origin of replication and intergenic region, which has been inserted at the NruI site in the pBR322 sequence. The presence of an f1 origin of replication permits generation of single-stranded DNA copies of the vector when transformed into appropriate strains of *E. coli* and superinfected with bacteriophage f1, which facilitates DNA sequencing of the vector and provides a basis for in vitro mutagenesis. To insert a cDNA, pIXY120 is digested with Asp718 which cleaves near the 3' end of the α-factor leader peptide (nucleotide 237) and, for example, NcoI which cleaves in the polylinker. The large vector fragment is then purified and ligated to a DNA fragment encoding the protein to be expressed.

To create a secretion vector for expressing human M-CSFγ, a cDNA fragment including the complete open reading frame encoding hM-CSFγ is cleaved with an appropriate restriction endonuclease proximal to the N-terminus of the mature protein. An oligonucleotide or oligonucleotides are then synthesized which are capable of ligation to the 5' and 3' ends of the hM-CSFγ fragment, regenerating any codons deleted in isolating the fragment, and also providing cohesive termini for ligation to pIXY120 to provide a coding sequence located in frame with respect to an intact α-factor leader sequence.

The resulting expression vectors are then purified and employed to transform a diploid yeast strain of *S. cerevisiae* (XV2181) by standard techniques, such as those disclosed in EPA 0165654, selecting for tryptophan prototrophs. The resulting transformants are cultured for expression of an hM-CSFγ protein as a secreted or extracted product. Cultures to be assayed for hM-CSFγ expression are grown in 20–50 ml of YPD medium (1% yeast extract, 2% peptone, 1% glucose) at 37° C. to a cell density of $1–5\times10^8$ cells/ml. To separate cells from medium, cells are removed by centrifugation and the medium filtered through a 0.45μ cellulose acetate filter prior to assay. Supernatants produced by the transformed yeast strain, or extracts prepared from disrupted yeast cells, are assayed for the presence of hG-CSFγ using binding assays as described above.

We claim:

1. An isolated DNA sequence comprising a nucleotide sequence encoding a functional biologically active human M-CSFγ protein.

2. An isolated DNA sequence according to claim 1, selected from the group consisting of:

(a) cDNA clones having the nucleotide sequence of the coding region of M-CSFγ plasmid pDCCSFγ;

(b) DNA sequences capable of hybridization to the clones of (a) under moderately stringent conditions and which encode functional biologically active M-CSFγ proteins; and

(c) DNA sequences which are degenerate as a result of the genetic code to the DNA sequences defined in (a) and (b) and which encode functional biologically active M-CSFγ proteins.

3. An isolated DNA sequence according to claim 1, wherein the DNA sequence encodes an M-CSFγ protein which comprises the sequence of amino acids 33–363 of FIG. 2 and does not include the sequence of amino acids 364–480 of FIG. 2.

4. An isolated DNA sequence according to claim 1, wherein the DNA sequence comprises the sequence of nucleotides 97–1090 of FIG. 2 and does not include the sequence of nucleotides 1091–1438 of FIG. 2.

5. An isolated DNA sequence according to claim 1, wherein the DNA sequence encodes a sequence of amino acids consisting essentially of the sequence of amino acids of M-CSFγ depicted in FIG. 2.

6. An isolated DNA sequence according to claim 1, wherein the DNA sequence consists essentially of the sequence of nucleotides encoding M-CSFγ depicted in FIG. 2.

7. A recombinant expression vector comprising a DNA sequence according to claim 1.

8. A recombinant expression vector comprising a DNA sequence according to claim 2.

9. A recombinant expression vector comprising a DNA sequence according to claim 3.

10. A recombinant expression vector comprising a DNA sequence according to claim 4.

11. A recombinant expression vector comprising a DNA sequence according to claim 5.

12. A recombinant expression vector comprising a DNA sequence according to claim 6.

13. A process for preparing a mammalian M-CSFγ protein comprising culturing a suitable host cell comprising a vector according to claim 7 under conditions promoting expression.

14. A process for preparing a human M-CSFγ protein comprising culturing a suitable host cell comprising a vector according to claim 11 under conditions promoting expression.

15. A process for preparing a mammalian M-CSFγ protein comprising culturing a suitable host cell comprising a vector according to claim 8 under conditions promoting expression.

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20 19 18 17 16 15 14

Table 2.10.3 High-Salt Solutions Used in Hybridization Analysis

Stock solution	Composition
20× SSC	3.0 M NaCl/0.3 M trisodium citrate
20× SSPE[a]	3.6 M NaCl/0.2 M NaH_2PO_4/0.02 M EDTA, pH 7.7
Phosphate solution[b]	1 M $NaHPO_4$, pH 7.2[c]

[a]SSC may be replaced with the same concentration of SSPE in all protocols.

[b]Prehybridize and hybridize with 0.5 M $NaHPO_4$ (pH 7.2)/1 mM EDTA/7% SDS [or 50% formamide/0.25 M $NaHPO_4$ (pH 7.2)/0.25 M NaCl/1 mM EDTA/7% SDS]; perform moderate-stringency wash in 40 mM $NaHPO_4$ (pH 7.2)/1 mM EDTA/5% SDS; perform high-stringency wash in 40 mM $NaHPO_4$ (pH 7.2)/1 mM EDTA/1% SDS.

[c]Dissolve 134 g $Na_2HPO_4 \cdot 7H_2O$ in 1 liter water, then add 4 ml 85% H_3PO_4. The resulting solution is 1 M Na^+, pH 7.2.

Designing washes for heterologous hybridization. Calculations of T_m become more critical if heterologous probing is being attempted. If the aim is to identify sequences that are merely related, not identical, to the probe (e.g., members of a multigene family, or a similar gene in a second organism), then it is useful to have an idea of the degree of mismatching that will be tolerated by a "moderate-" or "low-" stringency wash. The best way to approach this is to first establish the lowest temperature at which only homologous hybridization occurs with a particular SSC concentration. Then assume that 1% mismatching results in a 1°C decrease in the T_m (Bonner et al., 1973) and reduce the temperature of the final wash accordingly (for example, if sequences with ≥90% similarity with the probe are being sought, decrease the final wash temperature by 10°C). If the desired degree of mismatching results in a wash temperature of <45°C, then it is best to increase the SSC concentration so that a higher temperature can be used. Doubling the SSC concentration results in a ~17°C increase in T_m, so washes at 45°C in 0.1× SSC and 62°C in 0.2× SSC are roughly equivalent. Note that in these extreme cases it may also be necessary to reduce the hybridization temperature to as low as 45°C (aqueous solution) or 32°C (formamide solution).

This approach sometimes works extremely well (as shown when the heterologous targets are eventually sequenced), but the assumption that a 1% degree of mismatching reduces the T_m of a heteroduplex by 1°C is very approximate. Base composition and mismatch distribution influence the actual change in T_m, which can be anything between 0.5° and 1.5°C per 1% mismatch (Hyman et al., 1973). Unfortunately trial and error is the only alternative to the "rational" approach described here.

Other parameters relevant to hybridization analysis

Length of prehybridization and hybridization incubations. The protocols recommend prehybridization for 3 hr with nitrocellulose and 15 min for nylon membranes. Inadequate prehybridization can lead to high backgrounds, so these times should not be reduced. They can, however, be extended without problem.

Hybridizations are usually carried out overnight. This is a rather sloppy aspect of the procedure, because time can have an important influence on the result, especially if, as described above, an excess amount of a single-stranded probe is being used. The difficulties in assigning values to the parameters needed to calculate optimum hybridization time has led to the standard "overnight" incubation, which in fact is suitable for most purposes. The exception is when hybridization is being taken to its limits, for instance in detection of single-copy sequences in human DNA, when longer hybridization times (up to 24 hr) may improve sensitivity if a single-stranded probe is being used. Note that this does not apply to double-stranded probes, as gradual reannealing results in only minimal amounts of a double-stranded probe being free to hybridize after ~8 hr of incubation.

Formamide hybridization buffers. Formamide destabilizes nucleic acid duplexes, reducing the T_m by an average of 0.6°C per 1% formamide for a DNA-DNA hybrid (Meinkoth and Wahl, 1984) and rather less for a DNA-RNA hybrid (Casey and Davidson, 1977; Kafatos et al., 1979). It can be used at 50% concentration in the hybridization solution, reducing the T_m so that the incubation can be carried out at a lower temperature than needed with an aqueous solution. Originally formamide was used with nitrocellulose membranes as a means of prolonging their lifetime, as the

UNIT 2.6

Isolation and Purification of Large DNA Restriction Fragments from Agarose Gels

This unit describes methods for recovering and purifying DNA restriction fragments from agarose gels. The first basic protocol describes electroelution of the fragment of interest from standard agarose gels using buffer-filled dialysis bags, followed by concentration and purification using an Elutip column. This approach can be used effectively for fragments of all sizes from 50 to 20,000 bp. Electrophoresis directly onto NA-45 paper (second basic protocol) provides relatively high yields for fragments ≤2000 bp. Fragments ≥1000 bp can also be separated on low gelling/melting agarose gels and purified by phenol extraction (third basic protocol), β-agarase digestion of the gel (first alternate protocol), or via glass beads extraction (second alternate protocol). Removing linkers from a fragment using a column rather than a gel (third alternate protocol) is included, followed by a method for estimating DNA concentrations in solution (support protocol).

BASIC PROTOCOL

ELECTROELUTION FROM AGAROSE GELS

Following digestion with appropriate restriction enzymes, the DNA of interest is electrophoresed on a preparative agarose gel. The portion of the gel containing the restriction fragment to be purified is then physically removed from the remainder of the gel. This agarose slice is placed into a buffer-filled piece of dialysis tubing and again subjected to electrophoresis. The restriction fragment migrates out of the gel slice into the buffer, and the DNA is further purified and concentrated using an Elutip-d column. This procedure is effective with fragment sizes ranging from 50 to 20,000 bp, although for eluting fragments ≤2000 bp, the use of one of the alternate protocols (e.g., elution from NP-45 paper) is recommended.

Materials

For recipes, see Reagents and Solutions in this unit (or cross-referenced unit); for common stock solutions, see APPENDIX 2; for suppliers, see APPENDIX 4.

DNA encoding sequence of interest
Appropriate restriction enzymes and buffers (*UNIT 3.1*)
Ethidium bromide solution (*UNIT 2.5A*)
TAE buffer (*APPENDIX 2*)
Elutip high-salt solution (see recipe)
2.5 M NaCl
Elutip low-salt solution (see recipe)
100% and 70% ethanol
TE buffer, pH 8.0

Spectrapor 3 dialysis membrane tubing (11.5-mm diameter with MWCO of 3500; Baxter)
Elutip-d columns (Schleicher & Schuell)
Small syringe (e.g., 5-ml)

Additional reagents and equipment for restriction enzyme digestion (*UNIT 3.1*) and agarose gel electrophoresis and photography (*UNIT 2.5A*)

Choose and electrophorese a preparative agarose gel

1. Digest 0.1 to 25 µg DNA to completion with appropriate restriction enzyme(s).

The relative abundance of the DNA fragment to be isolated in the sample to be electrophoresed and the use to which this fragment will be put are the most important factors in determining the amount of DNA to be digested. For frequently used cloning

SYNOPSIS OF APPLICATION OF WRITTEN DESCRIPTION GUIDELINES

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SYNOPSIS OF APPLICATION OF WRITTEN DESCRIPTION GUIDELINES

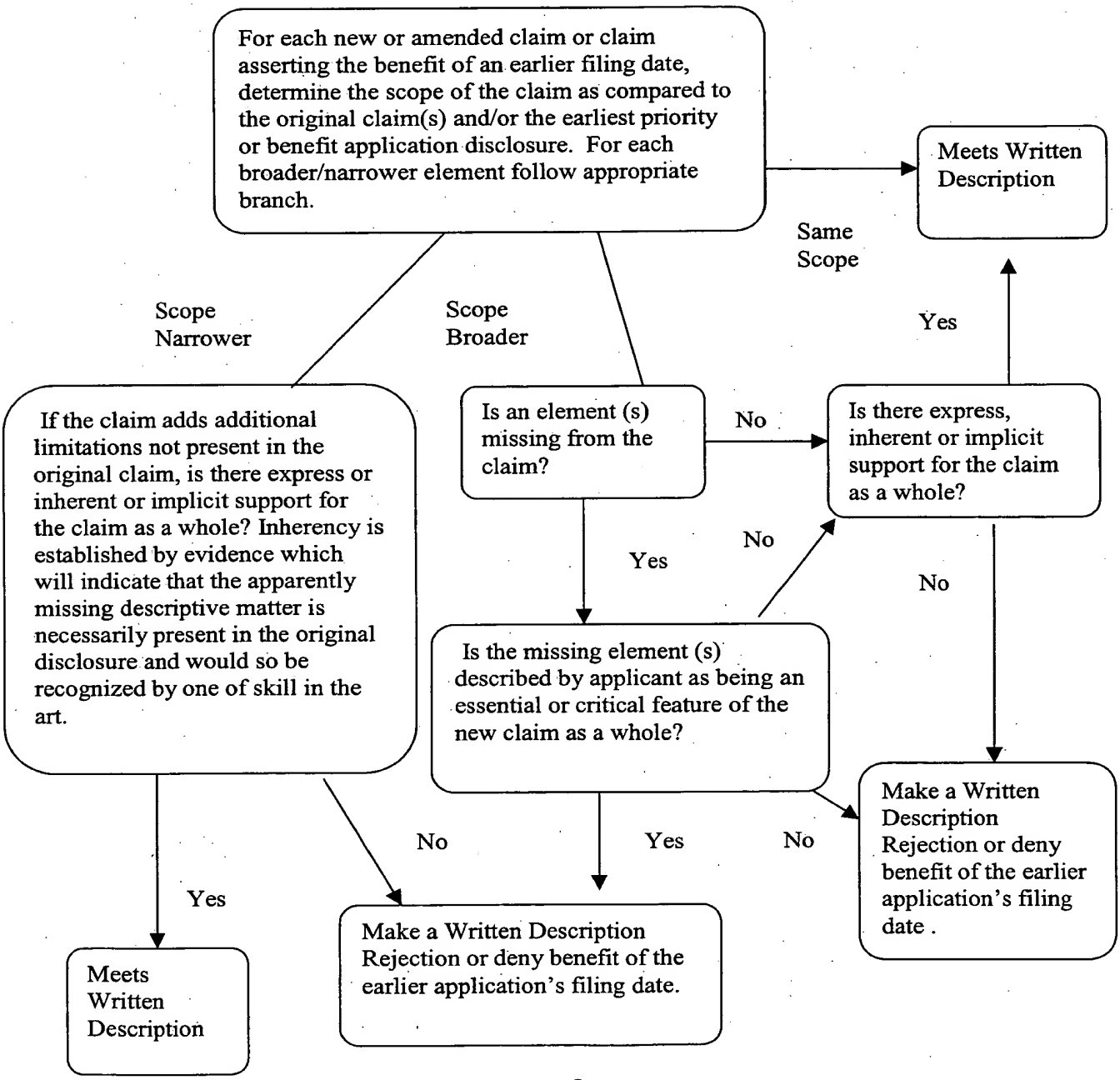
It is assumed at this point in the analysis that the specification has been reviewed and an appropriate search of the claimed subject matter has been conducted. It is also assumed that the examiner has identified which features of the claimed invention are conventional taking into account the body of existing prior art. There is a strong presumption that an adequate written description of the claimed invention is present in the specification as filed. If the examiner determines that the application does not comply with the written description requirement, the examiner has the initial burden, after a thorough reading and evaluation of the content of the application, of presenting evidence or reasons why a person skilled in the art would not recognize that the written description of the invention provides support for the claims. It should also be noted that the test for an adequate written description is separate and distinct from the test under the enablement criteria of 35 U.S.C. § 112 first paragraph. The absence of definitions or details for well-established terms or procedures should not be the basis of a rejection under 35 U.S.C. 112, para. 1, for lack of adequate written description. Limitations may not, however, be imported into the claims from the specification.

The following examples only describe how to determine whether the written description requirement of 35 U.S.C. 112, para. 1 is satisfied. Regardless of

the outcome of that determination, Office personnel must complete the patentability determination under all the relevant statutory provisions of Title 35 of the U.S. Code. Once Office personnel have concluded analysis of the claimed invention under all the statutory provisions, including 35 U.S.C. 101, 112, 102, and 103, they should review all the proposed rejections and their bases to confirm their correctness. Only then should any rejection be imposed in an Office action. The Office action should clearly communicate the findings, conclusions, and reasons which support them. When possible, the Office action should offer helpful suggestions on how to overcome rejections.

Written Description Amended or New Claims, or Claims Asserting the Benefit of an Earlier Filing Date

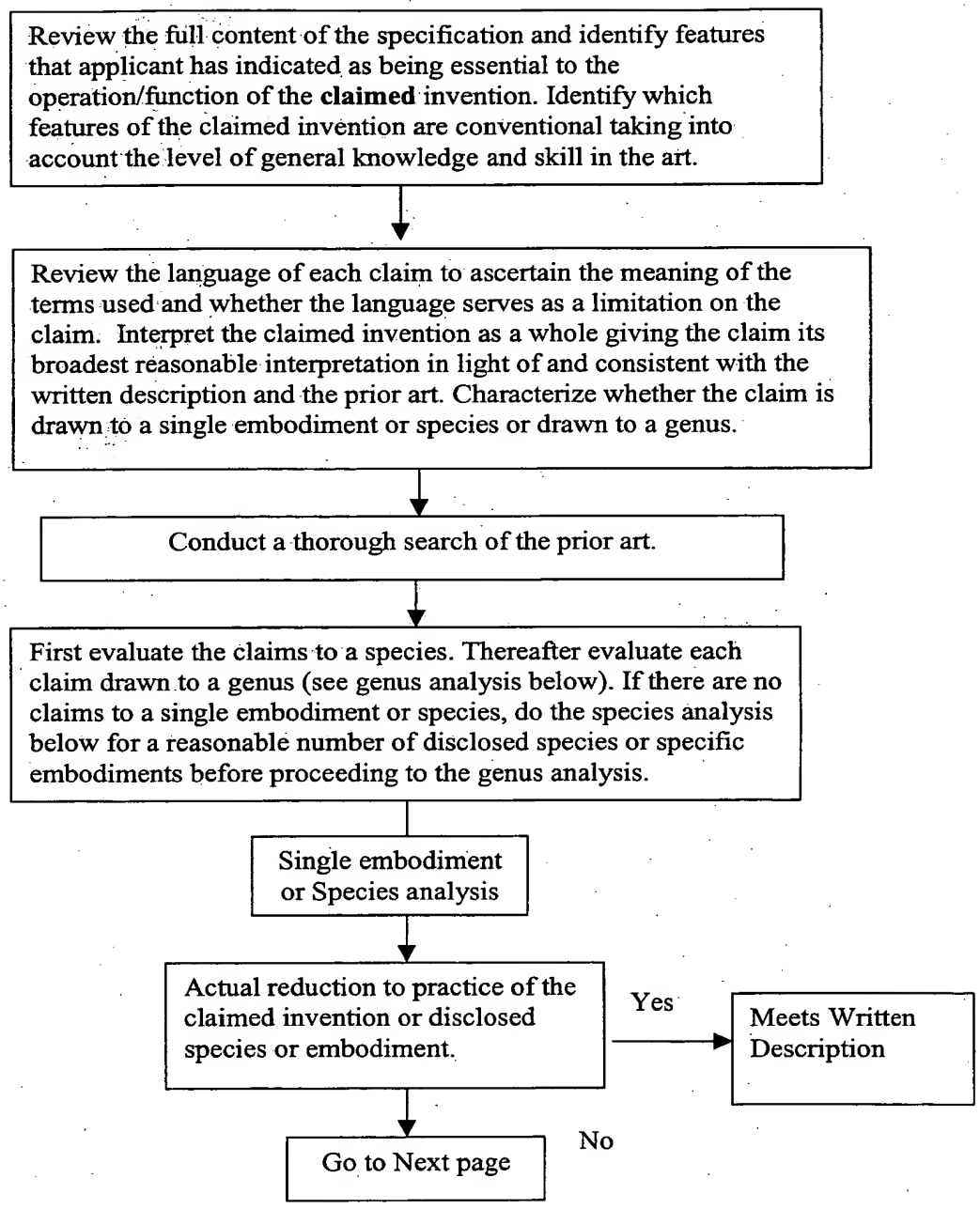
Decision Tree



Written Description

Original Claims

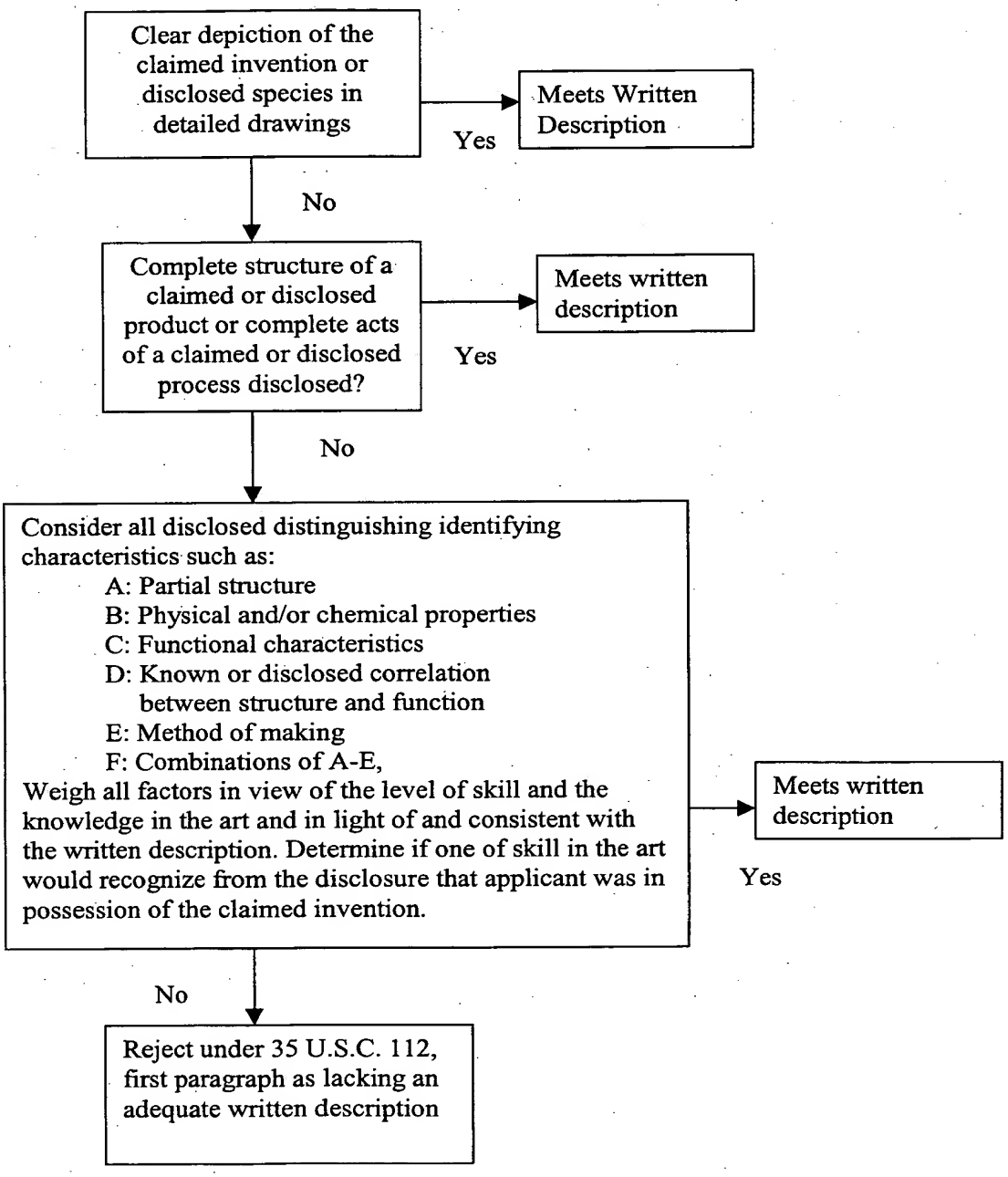
--Decision Tree--



Written Description

Original Claims

--Decision Tree--

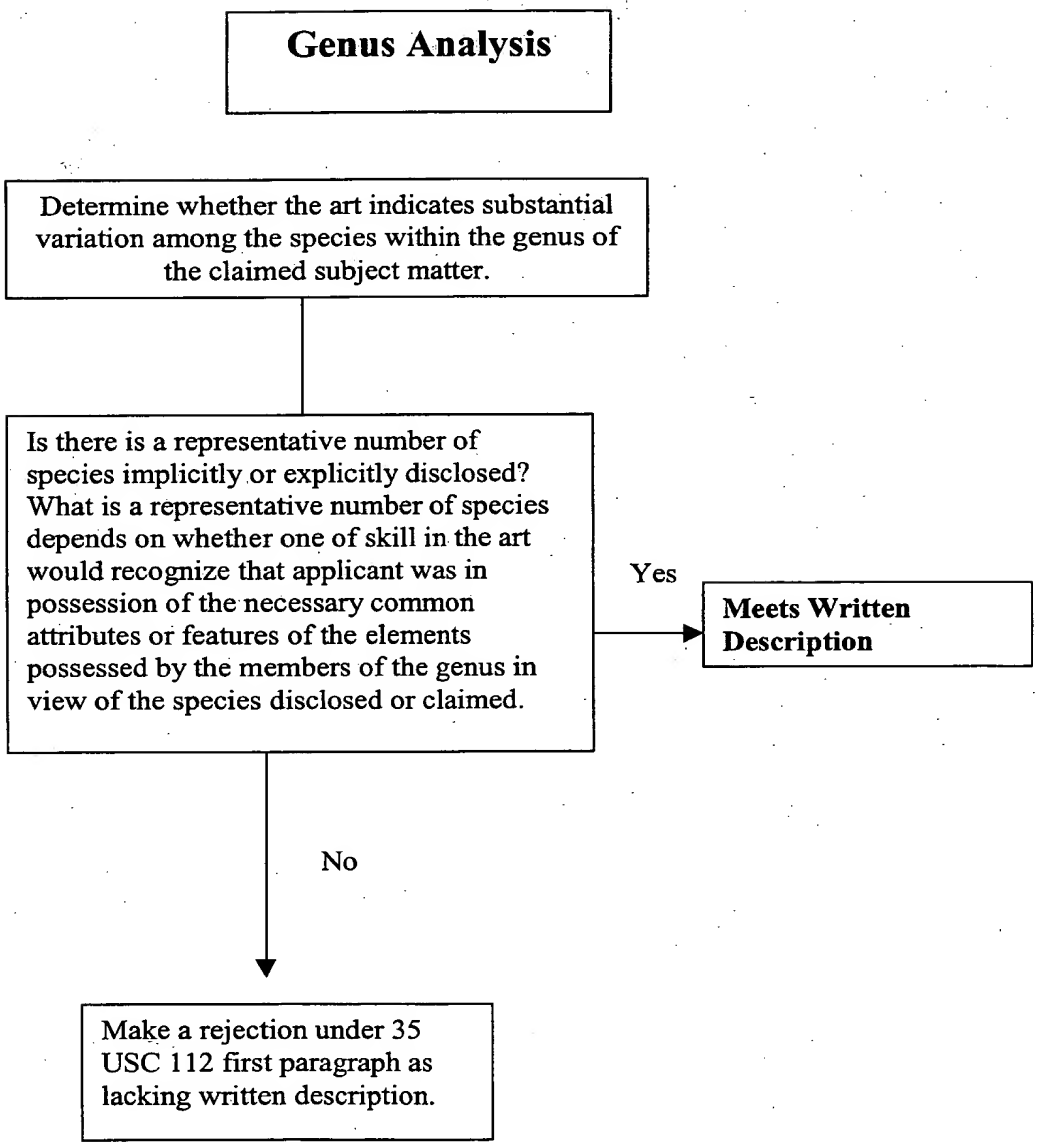


Written Description

Original Claims

Decision Tree

--Page 3--



WRITTEN DESCRIPTION TRAINING EXAMPLES

Example 1: Amended claims

Fact Pattern:

The specification is directed to a sectional sofa with a console between two reclining chairs, wherein control means for the reclining chairs are mounted on the console. The original disclosure clearly identifies the console as the only possible location for the controls, and provides for only the most minor variation in the location of the controls, e.g., the controls may be mounted on the top or side surfaces of the console or on the front wall. Additionally, the specification states that the purpose for the console is to house the controls. The original claims required the control elements to be present in the console. Applicant subsequently amends the claims to remove this limitation.

Amended Claim:

1. (Amended) A sectional sofa comprising:

a pair of reclining seats disposed in parallel relationship with one another in a double reclining seat sofa section, said double reclining seat sofa section being without an arm at one end whereby a second sofa section of the sectional sofa can be placed in abutting relationship with the end of the double reclining seat sofa section without an arm so as to form a continuation thereof,

each of said reclining seats having a backrest and seat cushion and movable between upright and reclined positions, said backrests and seat cushions of the pair of reclining sets lying in respective common planes when the seats are in the same positions,

a fixed console disposed in the double reclining seat sofa section between the pair of reclining seats and with the console and reclining seats together comprising a unitary structure, said console including an armrest portion for each of the reclining seats, said arm rests remaining fixed when the reclining seats move from one to another of their positions, and

a pair of control means [located upon the center console to enable each of the pair of reclining seats to move separately between the reclined and upright positions] mounted on the double reclining seat sofa section and each readily accessible to an occupant of its respective reclining seat and when actuated causing the respective reclining seat to move from the upright to the reclined position.

Analysis:

The amended claim is broader than the original claim in that the pair of control means is no longer required to be located on the center console. Thus, control means mounted on a center console is an element missing from the claim. The specification describes the location of the control means on the console as an essential feature of the claimed invention as a whole because the specification clearly identifies the console as the only possible location for the controls, and states that the purpose for the console is to house the controls.

Conclusion:

Reject the amended claim under 35 USC §112 first paragraph as lacking adequate written description.

Example 2: 35 USC 120 Priority

Fact Pattern:

The specification is directed to artificial hip sockets that include cup implants adapted for insertion into an acetabular, or hip, bone. The specification indicates that the shape of the cup is not important, as long as the implant can effectively function as an artificial hip socket. The application is a continuation in part of a parent application that describes an acetabular cup prosthesis wherein the cup is a trapezoid, a truncated cone, or of conical shape. All of these terms describe a conical cup. The parent specification also touts the criticality of a conical cup over all other shape cups.

A reference disclosing the claimed invention published between the filing date of the parent application and the instant application. Applicant asserts entitlement to the filing date of the parent application.

Claim:

1. An acetabular cup prosthesis comprising (1) a body extending generally longitudinally and terminating into front and rear surfaces, said front surface extending substantially transversely to said body; and (2) at least one fin for securing said cup to a prepared acetabulum cavity, said fin having a length extending generally longitudinally from said front surface toward said rear surface continuously along said body throughout the entire length of said fin, and said fin being configured so as to extend radially outwardly beyond the perimeter of said front surface and said body so as to engage with the cavity thereby securing said cup.

2. The prosthesis of claim 1, wherein the body has a generally conical outer surface.

Analysis:

Claim 1 in the instant application is directed to an acetabular cup prosthesis wherein the shape of the cup is not specifically defined (see element (1) of claim 1). The claim is broader than the disclosure in the parent application, which only describes a conical cup. Claim 1 is missing the element of a conical shape. This element is an essential or critical feature of the invention described in the parent application because the parent application only discloses a conical shape and the conical shape is described as critical over other shapes.

Claim 2 of the instant application is directed to an acetabular cup prosthesis wherein the cup has a generally conical outer surface. The claim is of the same scope as the invention described in the parent application.

Conclusion:

Reject claim 1 over the prior art reference, and indicate that the claim is not entitled to the benefit of the earlier application filing date.

Indicate that claim 2 is entitled to the benefit of the parent application filing date.

Note that if applicant had added the subject matter of claim 1 of this application to the parent application in an amendment, the claim would have been rejected under 35 U.S.C. 112, first paragraph as lacking an adequate written description.

Example 2A: Essential element missing from original claim

Fact Pattern:

The fact situation of example 2 above is similar to the fact situation of the instant example, however, there is no parent application in this example.

The specification is directed to artificial hip sockets that include cup implants adapted for insertion into an acetabular, or hip, bone. The specification indicates that the shape of the cup is critical to permit the implant to effectively function as an artificial hip socket. The application describes an acetabular cup prosthesis wherein the cup is a trapezoid, a truncated cone, or of conical shape. All of these terms describe a conical cup. The specification also touts the criticality of a conical cup.

Claims: Same as claims 1 and 2 of example 2 above.

Analysis:

Claim 1 in the instant application is directed to an acetabular cup prosthesis wherein the shape of the cup is not specifically defined (see element (1) of claim 1). The claim is broader than the disclosure in the instant application that only describes a conical cup. Claim 1 is missing the element of a conical shape. A review of the specification indicates that a cup implant having a shape which can effectively function as an artifical hip socket is critical to the operation/function of the claimed invention. The application discloses a conical shape cup and the conical shape is described as critical over other shapes. The specification indicates that the invention **as claimed** will not function in its intended manner without the specific cup

shape. Therefore this element is essential to the function/operation of the invention.

Claim 1 is directed to a genus. There is no actual reduction to practice or clear depiction of the claimed invention in detailed drawings; however, the complete structure of a species of the claimed prosthesis (with conical shape) is disclosed. The disclosed species is not representative of the genus because the specification indicates that without the conical shape the invention will not operate as intended. Therefore, applicant was not in possession of the necessary common attributes of the elements possessed by the members of the genus. A written description rejection should be made in this situation.

Example 2B: A preferred element missing from original claim

Fact Pattern:

The fact situation of example 2B is similar to example 2A above except that in this example the shape of the conical cup is described as being preferred.

The specification is directed to artificial hip sockets that include cup implants adapted for insertion into an acetabular, or hip, bone. The specification indicates that the shape of the cup must permit the implant to effectively function as an artificial hip socket. The application describes an acetabular cup prosthesis wherein the cup is preferably a trapezoid, a truncated cone, or of conical shape. All of these terms describe a conical cup. The specification emphasizes that a conical cup is the preferred embodiment.

Claims: Same as claims 1 and 2 of example 2 above.

Analysis:

Claim 1 in the instant application is directed to an acetabular cup prosthesis wherein the shape of the cup is not specifically defined (see element (1) of claim 1). The claim is broader than the disclosure in the instant application that only describes a conical cup. Claim 1 is missing the element of a conical shape. A review of the specification indicates that a cup implant having a conical shape is preferred but has no apparent bearing to the operation/function of the claimed invention. Therefore this element is not essential to the function or operation of the invention.

Claim 1 is directed to a genus. Although there is no actual reduction to practice or clear depiction of the claimed invention in detailed drawings, the complete structure of a species of the claimed prosthesis (with conical shape) is disclosed. The disclosed species is representative of the genus because there is a known correlation between the structure and the function of claimed invention and one of skill in the art would recognize that applicant was in possession of the necessary common attributes of the elements possessed by the members of the genus. The invention as claimed will function in its intended manner even without the specific cup shape. No written description rejection should be made in this situation.

Note: ***If the specification needs to be amended to be consistent with an original claim, see MPEP 608.01(o).***

Example 3: New claims

Fact Pattern:

The specification describes a form of computer technology called multi-threading. In essence, computers with multi-threading capabilities can switch between tasks with such rapidity that they appear to be performing two or more tasks at once. The specification describes one illustrative example in the specification wherein one of the program threads is an editor and another thread is a code processing routine in the form of a compiler. As the operator strikes keys at the keyboard, the compiler thread executes between each successive pair of keystrokes to process the entered source code concurrently with the editing operation. By the time the operator has finished entering or editing the code the compiler thread will have completed most of the required processing, thereby freeing the operator from lengthy periods of waiting for extensive code processing.

In this illustrative embodiment the interrupt operation of the central processor is periodically activated by a timer or clock. Each interrupt operation asynchronously preempts the executing compiler thread and passes control of the central processor to an interrupt service routine. The input port is then polled to test if a key has been struck at the keyboard. If not, the interrupt is terminated and control returns to the compiler thread. If polling the port reveals that a key has been struck then the interrupt service routine invokes the editor thread which takes control of the central processor to perform a character code entry or other edit operation. In addition to the description above, the application's abstract references an editor, compiler, interrupt means, and return means, and the "Object of the Invention" section

and the "Description of Prior Art" clearly discuss the importance of an editor and compiler.

The original claims required, *inter alia*, an editor, a compiler, an interrupt means and a return means. These elements are missing from new claim 20.

Claim:

20. A computer-readable disk memory having a surface formed with a plurality of binary patterns constituting a multithreaded application program executable by a desktop computer having a central microprocessor, a memory, means for loading said application program into a defined address space of said memory, and a clock-driven periodically-activated interrupt operation, said multithreaded program comprising

a plurality of sets of instructions with each set executable by said microprocessor,

a first of said sets of instructions executable to provide a first thread of execution having control of the central microprocessor,

said first thread of execution being periodically preempted in response to activations of an interrupt operation at predetermined fixed time intervals, and

a second of said sets of instructions executable to provide a second thread of execution to acquire control of the central microprocessor,

each of said threads having direct access to said program memory address space so as to provide fast efficient preemption of one thread by

another thread and switching of control of the central microprocessor back and forth among the threads at a rate so rapid that the threads execute effectively simultaneously.

Analysis:

Claim 20 is a new claim, which is broader in scope than the original claims. There are four elements missing from the claims (the editor, compiler, interrupt means, and return means). These missing elements are described by applicant as being an essential or critical feature of the claimed invention as a whole as evidenced by applicant's repeated reliance on the presence of these elements throughout the originally filed disclosure. Multiple sections within the application make clear that these four elements served integral functions in the overall invention.

Conclusion:

Reject claim 20 as lacking an adequate written description because four elements described as essential or critical are omitted. The omitted elements are: editor, compiler, interrupt means, and return means.

Example 4 : Original claim

Fact Pattern:

The invention is directed to a form of autopilot, described as a "heading lock," which enables a person to maintain directional control over a watercraft without constant manipulation of trolling motor controls. The preferred embodiment, as set forth in the written description and clearly depicted in detailed drawings, employs a compass mounted to the head of the "heading lock" unit, which monitors the direction of the thrust motor. The heading lock is coupled to the trolling motor; in a preferred embodiment, the heading lock is mechanically coupled to the trolling motor. The disclosure specifically notes that the direction of the thrust motor is considered to be the same as the direction of the boat since the trolling motor is mounted on the bow of the boat. The specification indicates that the electronic steering system continues to monitor the current heading of the thrust and also indicates that the heading detector continuously monitors the current heading of the boat. The term "heading" is used interchangeably throughout the written description to refer to both the direction of the trolling motor and the direction of the boat.

Claim:

1. A heading lock coupled to a trolling motor producing a thrust disposed to pull a watercraft, said heading lock comprising:

a steering motor coupled to said trolling motor, said steering motor being disposed to affect the orientation of said trolling motor in response to input signals;

a steering circuit electrically coupled to said steering motor, said steering circuit being disposed to generate said input signals to said steering motor in response to heading signals; and

a heading detector electrically coupled to said steering circuit, said heading detector being disposed to transmit said heading signals to said steering circuit.

Analysis:

Applicant has identified a heading lock comprising a steering system coupled to a trolling motor and a heading detector, as features essential to the operation of the claimed invention. Although the heading lock is preferably mechanically coupled to the trolling motor, the applicant does not describe the type of coupling as essential to the claimed invention as a whole. A search of the prior art shows that various means for coupling a heading lock to a trolling motor are conventional in the art. The claim is drawn to a single embodiment. Although there is no reduction to practice of the claimed invention, the claimed invention is clearly depicted in detailed drawings.

Conclusion:

The claim is adequately described.

Example 5: Flow Diagrams

Fact Pattern:

The specification is directed to a mechanism for controlling the mode of operation of a modem. A modem is used for modulating and demodulating signals, both analog and digital, over telephone lines. It has two modes: (1) a transparent mode, in which the modem performs the modulation-demodulation function, and (2) a command mode, in which the modem responds to predetermined commands and performs operations by executing a set of instructions stored in Read-Only-Memory (ROM) or firmware. An escape command tells the modem when to switch between transparent and command modes.

The application claims an improved mechanism for detecting an escape command by a modem. The decision making capability and timing means preferably reside in a microprocessor, preferably a Z-8 type microprocessor. The specification discloses logic flow diagrams and provides a detailed functional recitation that describes how to program computers to detect an escape command, but the specification does not provide a computer program listing with source code. The specification describes the escape sequence as one full second of no data, followed by the predetermined escape command, followed by another full second of no data.

Claim:

1. In a modem including a data input port for connecting said modem to a utilization device, and a telephone port for connecting said modem to a

telephone line, said modem being of the type having two distinct modes of operation:

(a) a transparent mode of operation for which said modem provides modulated signals to said telephone port in response to data signals provided to said data input port; and

(b) a command mode of operation for which said modem responds to said data signals provided to said data input port as instructions to said modem;

said modem including means defining a predetermined sequence of said data signals as an escape character; the improvement comprising:

timing means for detecting each occurrence of a passage of a predetermined period of time after provision of one of said data signals to said data input port; and

means, operative when said modem is in said transparent mode of operation, for detecting provision of said predetermined sequence of said data signals, and for causing said modem to switch to said command mode of operation, if and only if said predetermined sequence of data signals occurs contiguous in time with at least one said occurrence of said passage of said predetermined period of time during which none of said data signals are provided to said data input port.

Analysis:

After a review of the full content of the specification, the examiner finds that a modem having two modes of operation (transparent and

command), a timing means, and a means for detecting an escape sequence and causing the modem to switch from the transparent to the command mode are essential to the operation and function of the claimed invention. The specification does not describe a particular timing means or means for detecting the escape command and switching to the command mode. The claim is drawn to a genus. A search of the prior art indicates that the structure of the hardware required is conventional, and that one skilled in the art would know how to program a microprocessor to perform the necessary steps described in the specification. A review of the art indicates that there is no substantial variation among the species within the genus. Although no embodiments have been actually reduced to practice, a review of the specification shows that the claimed invention has been reduced to drawings in view of the detailed functional flow diagrams. Since the claimed invention is supported by conventional hardware structure and because there is a functional description of what the software does to operate the computer, there is sufficient description of the claimed invention. Disclosing a microprocessor capable of performing certain functions is sufficient to satisfy the requirement of section 112, first paragraph, when one skilled in the relevant art would understand what is intended and know how to carry it out.

Conclusion:

The claimed invention has been adequately described.

Biotechnology Examples

Example 6: Genes

Specification: The specification describes an isolated cDNA fragment (SEQ ID NO: 1; a 100mer) obtained from a human glioblastoma cDNA library. SEQ ID NO: 1 is asserted to be homologous to a known DNA molecule that encodes the extracellular domain of a glial specific G-coupled protein receptor whose function is associated with glial cell differentiation. The observed homology is sufficient to support a conclusion that SEQ ID NO: 1 would be glial specific. Further, it would be reasonable to infer that a G-coupled protein receptor encoded by a cDNA that comprised SEQ ID NO: 1 would be involved in the regulation of glial cell differentiation. In the description, applicant defines a "gene" as including naturally occurring regulatory elements and untranslated regions necessary and sufficient to mediate the expression of a cDNA comprising SEQ ID NO: 1. The specification describes methods for cloning nucleic acids that encode full-length glial specific G coupled protein receptors. The specification also discloses that SEQ ID NO: 1 can be used as a probe for identifying the presence of nucleic acids encoding glial specific G-coupled protein receptors in mammals. Glial specific G-coupled protein receptors are disclosed as useful in drug discovery methods to identify agents that regulate glial differentiation. The specification defines a probe as consisting of SEQ ID NO: 1 and between five to 10 additional nucleotides on either end of SEQ ID NO: 1.

Claim:

An isolated gene comprising SEQ ID NO: 1.

Analysis:

A review of the specification indicates that elements which are not particularly described, including regulatory elements and untranslated regions, are essential to the function of the claimed invention because applicant's definition of "gene" requires them. Additionally, SEQ ID NO: 1 is disclosed as being essential to the function of the claimed invention. The art indicates that the structure of genes with naturally occurring regulatory elements and untranslated regions is empirically determined. For example, the structural elements of "gene" mediating the expression of a particular protein in the liver may be different than the structural elements of the "gene" mediating the expression of the same protein in the brain. Therefore the structure of these elements which applicant considers as being essential to the function of the claim are not conventional in the art.

The claim is drawn to a genus, i.e., any gene which comprises SEQ ID NO: 1.

A search of the prior art indicates that SEQ ID NO: 1 is otherwise novel and unobvious, and no associated genomic clones have been identified.

There is no actual reduction to practice of the claimed invention, clear depiction of the claimed invention in the drawings or complete detailed description of the structure.

Considering all disclosed distinguishing identifying characteristics, there is a disclosure of partial structure (SEQ ID NO: 1) as well as the function of the gene as coding for a G-coupled protein receptor.

However, there is no known or disclosed correlation between this function and the structure of the non-described regulatory elements and untranslated regions of the gene. Furthermore, there is no additional disclosure of physical and/or chemical properties. Weighing all factors in view of the level of knowledge and skill in the art, one skilled in the art would not recognize from the disclosure that the applicant was in possession of the genus of genes which comprise SEQ ID NO: 1.

Conclusion:

Reject claim 1 under 35 USC 112 first paragraph as lacking an adequate written description. The examiner should make a rejection following a similar type of reasoning as that set forth above.

Note: Applicant may overcome this rejection by claiming a probe which consists essentially of SEQ ID NO: 1, since the specification teaches that a probe can have no more than 10 additional nucleic acid residues at either end of the molecule. The examiner should make an express determination that "consisting essentially of" admits of no more than 10 additional residues at either end of the molecule.

Example 7: EST

Specification: The specification discloses SEQ ID NO: 16 which is a partial cDNA. The specification does not address whether the cDNA crosses an exon/intron splice junction. The specification discloses that this sequence will specifically hybridize with the complement of the coding sequence of a gene of an infectious yeast. The presence of the nucleic acid detected by hybridization with the complement of the coding sequence is useful for identifying yeast infections. Example 1 of the specification describes an experiment where SEQ ID NO: 16 was determined following characterization of a cDNA clone isolated from a cDNA library.

Claim:

An isolated DNA comprising SEQ ID NO: 16.

Analysis:

A review of the full content of the specification indicates SEQ ID NO: 16 is essential to the operation and function of the claimed invention. The specification indicates that the presence of DNA that hybridizes with SEQ ID NO: 16 is indicative of a yeast infection.

A review of the language of the claim indicates that the claim is drawn to a genus, i.e., any nucleic acid that minimally contains SEQ ID NO: 16 within it including any full length gene which contains the sequence, any fusion constructs or cDNAs.

The search indicates that SEQ ID NO: 16 is a novel and unobvious sequence.

There is a single species explicitly disclosed (a molecule consisting of SEQ ID NO: 16 that is within the scope of the claimed genus).

There is actual reduction to practice of the disclosed species.

The disclosure of a single disclosed species may provide an adequate written description of a genus when the species disclosed is representative of the genus. The present claim encompasses full-length genes and cDNAs that are not further described. There is substantial variability among the species of DNAs encompassed within the scope of the claims because SEQ ID NO: 16 is only a fragment of any full-length gene or cDNA species. When reviewing a claim that encompasses a widely varying genus, the examiner must evaluate any necessary common attributes or features. In the case of a partial cDNA sequence that is claimed with open language (comprising), the genus of, e.g., "A cDNA comprising [a partial sequence]," encompasses a variety of subgenera with widely varying attributes. For example, a cDNA's principle attribute would include its coding region. A partial cDNA that did not include a disclosure of any open reading frame (ORF) of which it would be a part, would not be representative of the genus of cDNAs because no information regarding the coding capacity of any cDNA molecule would be disclosed. Further, defining "the" cDNA in functional terms would not suffice in the absence of a disclosure of structural features or elements of a cDNA that would encode a protein having a stated function.

A description of a genus of cDNAs may be achieved by means of a recitation of a representative number of cDNAs, defined by nucleotide sequence, falling within the scope of the genus or of a recitation of structural features common to the members of the genus, which features constitute a

substantial portion of the genus. Regents of the University of California v. Eli Lilly & Co., 119 F3d 1559, 1569, 43 USPQ2d 1398, 1406 (Fed. Cir. 1997).

Here, the specification discloses only a single common structural feature shared by members of the claimed genus, i.e., SEQ ID NO: 16. Since the claimed genus encompasses genes yet to be discovered, DNA constructs that encode fusion proteins, etc., the disclosed structural feature does not "constitute a substantial portion" of the claimed genus. Therefore, the disclosure of SEQ ID NO: 16 does not provide an adequate description of the claimed genus.

Weighing all factors, 1) partial structure of the DNAs that comprise SEQ ID NO: 16, 2) the breadth of the claim as reading on genes yet to be discovered in addition to numerous fusion constructs and cDNAs, 3) the lack of correlation between the structure and the function of the genes and/or fusion constructs; in view of the level of knowledge and skill in the art, one skilled in the art would not recognize from the disclosure that the applicant was in possession of the genus of DNAs which comprise SEQ ID NO: 16.

Conclusion: The written description requirement is not satisfied.

Caveat: *In situations where the specification indicates that the SEQ ID NO: is a full-length cDNA open reading frame and the claim* ***cannot*** *read on a gene, the claimed invention would meet the written description requirement.*

Example 8: <u>DNA fragment Encoding a Full Open Reading Frame (ORF)</u>

Specification: The specification discloses that a cDNA library was prepared from human kidney epithelial cells and 5000 members of this library were sequenced and open reading frames were identified. The specification discloses a Table that indicates that one member of the library having SEQ ID NO: 2 has a high level of homology to a DNA ligase. The specification teaches that this complete ORF (SEQ ID NO: 2) encodes SEQ ID NO: 3. An alignment of SEQ ID NO: 3 with known amino acid sequences of DNA ligases indicates that there is a high level of sequence conservation between the various known ligases. The overall level of sequence similarity between SEQ ID NO: 3 and the consensus sequence of the known DNA ligases that are presented in the specification reveals a similarity score of 95%. A search of the prior art confirms that SEQ ID NO: 2 has high homology to DNA ligase encoding nucleic acids and that the next highest level of homology is to alpha-actin. However, the latter homology is only 50%. Based on the sequence homologies, the specification asserts that SEQ ID NO: 2 encodes a ligase.

Claim 1: An isolated and purified nucleic acid comprising SEQ ID NO: 2.

Analysis:

A review of the full content of the specification indicates SEQ ID NO: 2 is essential to the operation and function of the claimed invention. The specification indicates that SEQ ID NO: 2 encodes a protein that would be expected to act as a DNA ligase.

A review of the language of the claim indicates that the claim is drawn to a genus, i.e., any nucleic acid that minimally contains SEQ ID NO: 2. The claim is drawn to a nucleic acid comprising a full open reading frame. The claimed nucleic acid does not read on a genomic sequence because full-length mammalian cDNAs would not be expected to contain introns or transcriptional regulatory elements such as promoters that are found in genomic DNA. The claim reads on the claimed ORF in any construct or with additional nucleic acid residues placed at either end of the ORF.

The search indicates that SEQ ID NO: 2 is a novel and unobvious sequence.

There is a single species explicitly disclosed (a molecule consisting of SEQ ID NO: 2 that is within the scope of the claimed genus).

There is actual reduction to practice of the disclosed species.

One of skill in the art can readily envisage nucleic acid sequences which include SEQ ID NO: 2 because e.g. SEQ ID NO: 2 can be readily embedded in known vectors. Although there may be substantial variability among the species of DNAs encompassed within the scope of the claim because SEQ ID NO: 2 may be combined with sequences known in the art,

e.g. expression vectors, the necessary common attribute is the ORF (SEQ ID NO: 2).

Weighing all factors including (1) that the full length ORF (SEQ ID NO: 2) is disclosed and (2) that any substantial variability within the genus arises due to addition of elements that are not part of the inventor's particular contribution, taken in view of the level of knowledge and skill in the art, one skilled in the art would recognize from the disclosure that the applicant was in possession of the genus of DNAs that comprise SEQ ID NO: 2.

Conclusion: The written description requirement is satisfied.

Example 9: Hybridization

Specification: The specification discloses a single cDNA (SEQ ID NO:1) which encodes a protein that binds to a dopamine receptor and stimulates adenylate cyclase activity. The specification includes an example wherein the complement of SEQ ID NO: 1 was used under highly stringent hybridization conditions (6XSSC and 65 degrees Celsius) for the isolation of nucleic acids that encode proteins that bind to dopamine receptor and stimulate adenylate cyclase activity. The hybridizing nucleic acids were not sequenced. They were expressed and several were shown to encode proteins that bind to a dopamine receptor and stimulate adenylate cyclase activity. These sequences may or may not be the same as SEQ ID NO: 1.

Claim:

An isolated nucleic acid that specifically hybridizes under highly stringent conditions to the complement of the sequence set forth in SEQ ID NO: 1,

wherein said nucleic acid encodes a protein that binds to a dopamine receptor and stimulates adenylate cyclase activity.

Analysis:

A review of the full content of the specification indicates that the essential feature of the claimed invention is the isolated nucleic acid that hybridizes to SEQ ID NO: 1 under highly stringent conditions and encodes a protein with a specific function. The art indicates that hybridization techniques using a known DNA as a probe under highly stringent conditions were conventional in the art at the time of filing.

The claim is drawn to a genus of nucleic acids all of which must hybridize with SEQ ID NO: 1 and must encode a protein with a specific activity.

The search of the prior art indicates that SEQ ID NO: 1 is novel and unobvious.

There is a single species disclosed (a molecule consisting of SEQ ID NO: 1) that is within the scope of the claimed genus.

There is actual reduction to practice of the disclosed species.

Now turning to the genus analysis, a person of skill in the art would not expect substantial variation among species encompassed within the scope of the claims because the highly stringent hybridization conditions set forth in the claim yield structurally similar DNAs. Thus, a representative number of species is disclosed, since highly stringent hybridization conditions in combination with the coding function of DNA and the level of

skill and knowledge in the art are adequate to determine that applicant was in possession of the claimed invention.

Conclusion: The claimed invention is adequately described.

Example 10: Process claim

Specification: The specification teaches that SEQ ID NO: 10 is an EST. The specification also teaches that SEQ ID NO: 10 is a chromosome marker and that any DNA which hybridizes under specified stringent conditions to SEQ ID NO: 10 will be useful as a marker for detecting the presence of Burkitt's lymphoma. The specification also teaches how to produce DNAs including genomic DNAs which hybridize to SEQ ID NO: 10 and isolation of said DNAs. The specification presents an example where a genomic DNA is probed with SEQ ID NO: 10 under the specified stringent conditions (6XSSC and 65 degrees Celsius) and the genomic DNA which hybridizes under these conditions is isolated and is sequenced. The sequence of this genomic clone is represented by SEQ ID NO: 11.

Claim:

Claim 1: A process for producing an isolated polynucleotide comprising hybridizing SEQ ID NO: 10 to genomic DNA in 6XSSC and 65° C and isolating the DNA polynucleotide detected with SEQ ID NO: 10.

Claim 2: An isolated DNA that hybridizes with SEQ ID NO: 10.

Analysis:

Claim 1:

A review of the full content of the specification indicates that the essential feature of the claimed invention is a process of obtaining a nucleic acid sequence which is identified by a probe that hybridizes to SEQ ID NO:10 and a polynucleotide that hybridizes with SEQ ID NO: 10. The

specification and the general state of the art indicate that the general process of producing nucleic acids through hybridization with probes was routine at the time of filing.

The claim is drawn to a genus i.e., a process of hybridizing to genomic DNA with SEQ ID NO: 10 and isolating the DNA which hybridizes under specific conditions to said sequence.

The search indicates that SEQ ID NO: 10 and SEQ ID NO: 11 are novel and unobvious sequences. Therefore, under the examination guidelines of *In re Ochiai* and *In re Brouwer*, the method of making a novel and unobvious product is also novel and unobvious.

The specification presents an example where a single species has been reduced to practice, i.e., isolation of SEQ ID NO: 11 based on hybridization with SEQ ID NO: 10. Therefore the disclosed species within the genus has been adequately described. Now turning to the genus analysis, the art indicates that there is no substantial variation within the genus because of the stringency of hybridization conditions which yields structurally similar molecules. The single disclosed species is representative of the genus because reduction to practice of this species, considered along with the defined hybridization conditions and the level of skill and knowledge in the art, are sufficient to allow the skilled artisan to recognize that applicant was in possession of the necessary common attributes or features of the elements possessed by the members of the genus.

Claim 2:

The claim is drawn to a genus of nucleic acids, all of which must hybridize to SEQ ID NO: 10. The claim does not specify any stringency conditions. The claim is broad and reads on virtually any nucleic acid.

There is a species disclosed, SEQ ID NO: 11. The art indicates that there is substantial variation within the genus because the lack of stringency of hybridization conditions would be expected to yield structurally unrelated nucleic acid molecules. The single disclosed species is not representative of the genus because there is no structural attribute or feature that is common to the members of the genus.

Conclusion:

Claim 1 is adequately described.

Claim 2 should be rejected as lacking adequate written description following the analysis described above.

Note: Applicant may overcome the written description rejection of the product by, for example, substituting claim 2 with a product by process claim such as the one below.

Claim 2. *The isolated DNA polynucleotide prepared according to the process of claim 1.*

Example 11: Allelic Variants

Specification: The specification discloses a DNA, SEQ ID NO: 1, said to encode a cell surface receptor for adenovirus. The cell surface receptor is designated protein X and its sequence is given as SEQ ID NO:2. The specification states that the invention includes alleles of the DNA that include single nucleotide polymorphisms (SNPs). No allelic sequence information is disclosed, but the specification states that allelic variants of SEQ ID NO: 1 can be obtained, e.g., by hybridizing SEQ ID NO: 1 to a DNA library made from the species of organism that yielded SEQ ID NO: 1.

Claims:

1. An isolated DNA that encodes protein X (SEQ ID NO: 2).

2. An isolated allele of the DNA according to claim 1, which allele encodes protein X (SEQ ID NO: 2).

3. An isolated allele of SEQ ID NO: 1.

Analysis:

Claim 1:

Claim 1 is drawn to the genus of DNAs that encode amino acid sequence SEQ ID NO:2, i.e., all sequences degenerately related by a genetic code table to SEQ ID NO:1. Although only one specie within the genus is disclosed, SEQ ID NO:1, a person of skill in the art could readily envision all the DNAs degenerate to SEQ ID NO:1 by using a genetic code table. One of skill in the art would conclude that applicant was in possession of the

genus based on the specification and the general knowledge in the art concerning a genetic coding table.

Claim 2:

Claim 2 is drawn to a subgenus of allelic DNAs that encode amino acid sequence SEQ ID NO: 2. The specification does not provide any particular definition for the term allele. In this circumstance, the meaning of the term is the ordinary usage in the art. The ordinary meaning of the term allele is one of two or more alternate forms of a gene occupying the same locus in a particular chromosome or linkage structure and differing from other alleles of the locus at one or more mutational sites. See, Rieger et al., *Glossary of Genetics* (1991), p. 16. The alleles in claim 2 are "strictly neutral" because they encode identical proteins, and make no difference to phenotype. See, Rieger et al., p. 17. Although the standard definition refers to genomic sequences and the claims are directed to DNAs, a reasonable interpretation is that the claim is directed to DNAs that include naturally occurring mutational site(s).

The specification discloses only one allele within the scope of the genus: SEQ ID NO:1. The specification proposes to discover other members of the genus by using a hybridization procedure. There is no description of the mutational sites that exist in nature, and there is no description of how the structure of SEQ ID NO: 1 relates to the structure of any strictly neutral alleles. The general knowledge in the art concerning alleles does not provide any indication of how the structure of one allele is representative of unknown alleles. The nature of alleles is that they are variant structures, and in the present state of the art the structure of one does

not provide guidance to the structure of others. The common attributes of the genus are not described. One of skill in the art would conclude that applicant was not in possession of the claimed genus because a description of only one member of this genus is not representative of the variants of the genus and is insufficient to support the claim.

Claim 3:

Claim 3 is drawn to the genus including all DNA alleles of SEQ ID NO: 1. The specification does not provide any particular definition for the term allele. In this circumstance, the meaning of the term is the ordinary usage in the art. The ordinary meaning of the term allele is one of two or more alternate forms of a gene occupying the same locus in a particular chromosome or linkage structure and differing from other alleles of the locus at one or more mutational sites. See, Rieger et al., *Glossary of Genetics* (1991), p. 16. The Rieger reference discloses that there are at least seven different kinds of allele in addition to the "strictly neutral" type discussed above for Claim 2. See, Rieger, pp. 16-17 (amorphs, hypomorphs, hypermorphs, antimorphs, neomorphs, isoalleles, and unstable alleles). The alleles are distinguished by the effect their different structures have on phenotype. According to Rieger, alleles may differ functionally according to their distinct structures. For example, they may differ in the amount of biological activity the protein product may have, may differ in the amount of protein produced, and may even differ in the kind of activity the protein product will have.

The specification discloses only one allele within the scope of the genus: SEQ ID NO:1. The specification proposes to discover other

members of the genus by using a hybridization procedure. There is no description of the mutational sites that exist in nature, and there is no description of how the structure of SEQ ID NO: 1 relates to the structure of different alleles. In addition, according to the standard definition, the genus includes members that would be expected to have widely divergent functional properties. The general knowledge in the art concerning alleles does not provide any indication of how the structure of one allele is representative of other unknown alleles having concordant or discordant functions. The common attributes of the genus are not described and the identifying attributes of individual alleles, other than SEQ ID NO:1, are not described. The nature of alleles is that they are variant structures where the structure and function of one does not provide guidance to the structure and function of others. According to these facts, one of skill in the art would conclude that applicant was not in possession of the claimed genus because a description of only one member of this genus is not representative of the variants of the genus and is insufficient to support the claim.

Conclusions:

Claim 1:

Claim 1 should not be rejected under the written description requirement.

Claim 2:

Claim 2 should be rejected under the written description requirement. An analysis similar to the one set forth above could be used. Since the Office has the burden of presenting evidence to support its position, see

MPEP 2163.04, a reference should be relied on as authority for the Office's interpretation of the claim term "allele."

Claim 3:

Claim 3 should be rejected under the written description requirement. An analysis similar to the one set forth above could be used. Since the Office has the burden of presenting evidence to support its position, see MPEP 2163.04, a reference should be relied on as authority for the Office's interpretation of the claim term "allele."

For the rejections of claims 2 and 3, the Office interpretation of "allele" should be supported by a reference, rather than by taking "notice," because the interpretation is the principle evidence supporting the rejection. See MPEP 2144.03 (For further views on official notice, see *In re Ahlert*, 424 F.2d 1088, 1091 165 USPQ 418, 420 - 421 (CCPA 1970) ("[A]ssertions of technical facts in areas of esoteric technology must always be supported by citation of some reference work" and "allegations concerning specific `knowledge' of the prior art, which might be peculiar to a particular art should also be supported." Furthermore the applicant must be given the opportunity to challenge the correctness of such assertions and allegations. "The facts so noticed serve to `fill the gaps' which might exist in the evidentiary showing" and should not comprise the principle evidence upon which a rejection is based.); see also, *In re Barr*, 444 F.2d 588, 170 USPQ 330 (CCPA 1971) (scientific journal references were not used as a basis for taking judicial notice that controverted phrases were art - recognized because the court was not sure that the meaning of the term at issue was indisputable among reasonable men); *In re Eynde*, 480 F.2d 470, 178 USPQ

470,474 (CCPA 1973) ("The facts constituting the state of the art are normally subject to the possibility of rational disagreement among reasonable men and are not amenable to the taking of [judicial] notice.").)

Example 12: Bioinformatics

Specification: The specification discloses a process for identifying and selecting biological compounds that are present in a biological system in a tissue specific manner. In the disclosed process the expression level of a set of compounds is quantitatively determined in multiple tissues within an organism. The expression level data is then graphically displayed in such a manner that compounds that are differentially expressed are easily identified. An artisan interested in identifying a compound that is expressed at a high level in one tissue and at a different level in a second tissue may easily select compounds that are expressed in a tissue specific manner based on the displayed information. The specification indicates that the compounds to be detected encompass DNA, RNA and proteins as well as metabolites. The specification does not provide any particular examples, but discloses that the expression levels can be determined by any analytical method consistent with the class of compounds being detected. This type of measurement requires actual physical steps.

Claim:

A computer-implemented method of selecting tissue specific compounds, said method comprising the steps of:

(a) analyzing the expression level of compounds in a first and second tissue and obtaining expression level data for each of said compounds;

(b) inputting the expression level data obtained in step a) into a computer;

(c) displaying a first axis corresponding to the expression level of each of said compounds in said first tissue;

(d) displaying a second axis substantially perpendicular to said first axis, said second axis corresponding to the expression level data of each of said compound in said second sample

(e) displaying a mark at a position, wherein said position is selected relative to said first axis in accordance with an expression level of each of said compound in said first sample and relative to said second axis in accordance with the expression of said compound in said second sample; and

(f) selecting a compound of interest based on the position of the mark.

Analysis:

A review of the full content of the specification indicates that obtaining, inputting, and displaying the expression level of compounds is essential to the operation of the claimed invention.

A search of the prior art indicates that obtaining the expression level data of compounds is conventional in the art, and that data display devices and associated support algorithms are well known in the art.

A review of the claim indicates that the claim is drawn to a generic environment for the display of compounds in a tissue specific manner.

Since there is no species claimed or disclosed, the claim is analyzed as a claim drawn to a single embodiment. There is no actual reduction to practice of the claimed invention, or clear depiction of the claimed invention

in detailed drawings. However, reading the specification in light of the knowledge and level of skill in the art, the specification discloses the complete steps of the claimed process. See In re Hayes Microcomputer Products Inc. Patent Litigation, 982 F2d. 1527, 1534-35, 25 USPQ2d 1241, 1246 (Fed. Cir. 1992), where the court stated,

> One skilled in the art would know how to program a microprocessor to perform the necessary steps desired in the specification. Thus, an inventor is not required to describe every detail of his invention. An applicant's disclosure obligation varies according to the art to which the invention pertains.

In this fact situation, the art is sufficiently developed so as to put one of skill in the art in possession of the complete steps of the process. In other words, one skilled in the relevant art would understand what is intended by the claimed invention and know how to carry it out.

Conclusion: There is adequate written description for what is claimed.

Example 13: Protein Variant

Specification: The specification describes a protein isolated from liver. A working example shows that the isolated protein was sequenced and determined to consist of SEQ ID NO: 3. The isolated protein was additionally characterized as being 65 kD in molecular weight and having tumor necrosis activity. The specification states that the invention provides variants of SEQ ID NO: 3 having one or more amino acid substitutions, deletions, insertions and/or additions. No further description of the variants is provided. The specification indicates that procedures for making proteins with substitutions, deletions, insertions and/or additions are routine in the art. The specification does not define when a protein ceases to be a variant of SEQ ID NO: 3.

Claims:

1. An isolated protein having SEQ ID NO: 3.

2. An isolated variant of the protein of claim 1.

Analysis:

Claim 1:

A search of the prior art indicates that SEQ ID NO: 3 is novel and nonobvious. The claim is directed to a genus of proteins that comprise SEQ ID NO: 3. One member of the genus, SEQ ID NO: 3, is described by a complete structure.

There is relatively little variation among the species within the genus because each member of the genus shares SEQ ID NO: 3 as a necessary common feature. The single disclosed example is representative of the claimed genus because taken in view of the general knowledge in the art, the disclosure is sufficient to show that one of skill in the art would conclude that applicant was in possession of the claimed genus.

Claim 2:

This is a genus claim. According to the specification, the term variant means a protein having one or more amino acid substitutions, deletions, insertions and/or additions made to SEQ ID NO: 3. The specification and claim do not indicate what distinguishing attributes shared by the members of the genus. The specification and claim do not place any limit on the number of amino acid substitutions, deletions, insertions and/or additions that may be made to SEQ ID NO: 3. Thus, the scope of the claim includes numerous structural variants, and the genus is highly variant because a significant number of structural differences between genus members is permitted. Although the specification states that these types of changes are routinely done in the art, the specification and claim do not provide any guidance as to what changes should be made. Structural features that could distinguish compounds in the genus from others in the protein class are missing from the disclosure. No common structural attributes identify the members of the genus. The general knowledge and level of skill in the art do not supplement the omitted description because specific, not general, guidance is what is needed. Since the disclosure fails to describe the common attributes or characteristics that identify members of the genus, and because the genus is highly variant, SEQ ID NO: 3 alone is insufficient to

describe the genus. One of skill in the art would reasonably conclude that the disclosure fails to provide a representative number of species to describe the genus. Thus, applicant was not in possession of the claimed genus.

Conclusions:

Claim 1:

The claimed subject matter is adequately described. A rejection under the written description requirement should not be entered.

Claim 2:

The claimed subject matter is not supported by an adequate written description because a representative number of species have not been described. A rejection under the written description requirement, relying on the analysis set out above, should be entered.

Example 14: Product by Function

Specification: The specification exemplifies a protein isolated from liver that catalyzes the reaction of A $\longrightarrow$ B. The isolated protein was sequenced and was determined to have the sequence as set forth in SEQ ID NO: 3. The specification also contemplates but does not exemplify variants of the protein wherein the variant can have any or all of the following: substitutions, deletions, insertions and additions. The specification indicates that procedures for making proteins with substitutions, deletions, insertions and additions is routine in the art and provides an assay for detecting the catalytic activity of the protein.

Claim:

A protein having SEQ ID NO: 3 and variants thereof that are at least 95% identical to SEQ ID NO: 3 and catalyze the reaction of A $\longrightarrow$ B.

Analysis:

A review of the full content of the specification indicates that a protein having SEQ ID NO: 3 or variants having 95% identity to SEQ ID NO: 3 and having catalytic activity are essential to the operation of the claimed invention. The procedures for making variants of SEQ ID NO: 3 are conventional in the art and an assay is described which will identify other proteins having the claimed catalytic activity. Moreover, procedures for making variants of SEQ ID NO: 3 which have 95% identity to SEQ ID NO: 3 and retain its activity are conventional in the art.

A review of the claim indicates that variants of SEQ ID NO: 3 include but are not limited to those variants of SEQ ID NO: 3 with substitutions, deletions, insertions and additions; but all variants must possess the specified catalytic activity and must have at least 95% identity to the SEQ ID NO: 3. Additionally, the claim is drawn to a protein which **comprises** SEQ ID NO: 3 or a variant thereof that has 95% identity to SEQ ID NO: 3. In other words, the protein claimed may be larger than SEQ ID NO: 3 or its variant with 95% identity to SEQ ID NO: 3. It should be noted that "having" is open language, equivalent to "comprising".

The claim has two different generic embodiments, the first being a protein which comprises SEQ ID NO: 3 and the second being variants of SEQ ID NO: 3. There is a single species disclosed, that species being SEQ ID NO: 3.

A search of the prior art indicates that SEQ ID NO: 3 is novel and unobvious.

There is actual reduction to practice of the single disclosed species. The specification indicates that the genus of proteins that must be variants of SEQ ID NO: 3 does not have substantial variation since all of the variants must possess the specified catalytic activity and must have at least 95% identity to the reference sequence, SEQ ID NO: 3. The single species disclosed is representative of the genus because all members have at least 95% structural identity with the reference compound and because of the presence of an assay which applicant provided for identifying all of the at least 95% identical variants of SEQ ID NO: 3 which are capable of the specified catalytic activity. One of skill in the art would conclude that

applicant was in possession of the necessary common attributes possessed by the members of the genus.

Conclusion: The disclosure meets the requirements of 35 USC §112 first paragraph as providing adequate written description for the claimed invention.

Example 15: Antisense

Specification: The specification discloses a messenger RNA sequence, SEQ ID NO: 1, which encodes human growth hormone. The specification states that the invention includes antisense molecules that inhibit the production of human growth hormone. The specification describes an art-recognized method of screening for antisense molecules that is called "gene walking." Gene walking is said to involve obtaining antisense oligonucleotides that are complementary to the target sequence.

Claim:

An antisense oligonucleotide complementary to a messenger RNA having SEQ ID NO: 1 and encoding human growth hormone, wherein said oligonucleotide inhibits the production of human growth hormone.

Analysis:

A review of the full content of the specification indicates that the complement of SEQ ID NO: 1 is essential to the operation of the claimed invention. The general knowledge in the art is that any full-length complement of a target mRNA inhibits the function of the mRNA and is therefore an antisense oligonucleotide. Thus, one of skill in the art would view applicant's disclosure of a coding sequence, with the statement that the invention includes antisense oligonucleotides, as an implicit disclosure that the full-length complement of SEQ ID NO: 1 is an antisense oligonucleotide.

It is generally accepted in the art that oligonucleotides complementary to a messenger RNA, including fragments of the full-length complement, have antisense activity when they match accessible regions on the target mRNA. Generally, the closer the complementary fragment is to full length, the greater the likelihood it will have antisense activity. In addition, oligos that retain complementarity to the Shine-Delgarno sequence usually have antisense activity.

The claim is drawn to the genus of antisense molecules that inhibit the production of human growth hormone encoded by SEQ ID NO: 1. There is a single species described with a complete structure, i.e., the full-length complement of SEQ ID NO: 1. In addition to the full-length complement, the genus includes fragments of the complement that retain antisense activity.

The procedures for making oligonucleotide fragments of the SEQ ID NO: 1 complement are conventional, e.g., any specified fragment can be ordered from a commercial synthesizing service. The procedures for screening for antisense activity are also conventional, and the specification describes the assay needed to do gene walking. The experience accumulated in the art with gene walking is that numerous regions of a target are accessible, that these regions are identified routinely, and that antisense oligonucleotides are complementary to these accessible regions. The full-length complement and longer fragments match multiple accessible regions; shorter fragments match fewer accessible regions.

When considering the distinguishing characteristics of the claimed invention, the sequence provided in the specification defines and limits the

structure of any effective antisense molecules. The specification also teaches the functional characteristics of the claimed invention as well as a routine art recognized method of making and screening for the claimed invention. Considering the specification's disclosure of:

(1) the sequence (SEQ ID NO: 1) which defines and limits the structure of any effective antisense molecules such that one skilled in the art would be able to immediately envisage members of the genus embraced by the claim, and

(2) the functional characteristics of the claimed invention as well as a routine art-recognized method of screening for antisense molecules which provide further distinguishing characteristics of the claimed invention, along with

(3) the general level of knowledge and skill in the art,

one skilled in the art would conclude that applicant was in possession of the invention.

Conclusion: The claimed invention is adequately described.

Example 16: Antibodies

Specification: The specification teaches that antigen X has been isolated and is useful for detection of HIV infections. The specification teaches antigen X as purified by gel filtration and provides characterization of the antigen as having a molecular weight of 55 KD. The specification also provides a clear protocol by which antigen X was isolated. The specification contemplates but does not teach in an example antibodies which specifically bind to antigen X and asserts that these antibodies can be used in immunoassays to detect HIV. The general knowledge in the art is such that antibodies are structurally well characterized. It is well known that all mammals produce antibodies and they exist in five isotypes, IgM, IgG, IgD, IgA and IgE. Antibodies contain an effector portion which is the constant region and a variable region that contains the antigen binding sites in the form of complementarity determining regions and the framework regions. The sequences of constant regions as well as the variable regions subgroups (framework regions) from a variety of species are known and published in the art. It is also well known that antibodies can be made against virtually any protein.

Claim: An isolated antibody capable of binding to antigen X.

Analysis:

A review of the full content of the specification indicates that antibodies which bind to antigen X are essential to the operation of the claimed invention. The level of skill and knowledge in the art of antibodies at the time of filing was such that production of antibodies against a well-

characterized antigen was conventional. This is a mature technology where the level of skill is high and advanced.

The claim is directed to any antibody which is capable of binding to antigen X.

A search of the prior art indicates that antigen X is novel and unobvious.

Considering the routine art-recognized method of making antibodies to fully characterized antigens, the well defined structural characteristics for the five classes of antibody, the functional characteristics of antibody binding, and the fact that the antibody technology is well developed and mature, one of skill in the art would have recognized that the spectrum of antibodies which bind to antigen X were implicitly disclosed as a result of the isolation of antigen X.

Conclusion: The disclosure meets the requirement under 35 USC 112 first paragraph as providing an adequate written description of the claimed invention.

Example 17: Genus-species with widely varying species

Specification: The specification discloses the rat cDNA sequences for proinsulin and pre-proinsulin and a method for determining the corresponding human and other mammalian insulin cDNA sequences. However, the specification does not disclose any actual cDNA sequence other than the rat proinsulin and pre-proinsulin sequence. The specification discloses that one human proinsulin amino acid (but not cDNA) sequence was known at the time of filing. The art recognized that the sequence of human insulin proteins, and therefore also cDNAs, would probably vary among individuals. The specification also discloses that pre-proinsulin is post translationally modified to form proinsulin, and that proinsulin is cleaved to form insulin.

Claims:

Claim 1. An isolated mammalian cDNA encoding insulin.

Claim 2. The isolated cDNA of claim 1 wherein the mammalian cDNA is human.

Analysis: The examiner should analyze claim 2 first because it is drawn to a subgenus of the genus of claim 1.

Claim 2:

A review of the full content of the specification indicates that human cDNA molecules that encode insulin are essential to the operation/function of the invention.

Claim 2 is directed to a genus of human cDNA which encodes insulin.

There is no species of human insulin cDNA disclosed.

Based upon art published after applicant's filing date there is expected to be variation among the species of cDNA which encode human insulin because the sequence of human insulin proteins, and therefore also human insulin cDNAs, would be expected to vary among individuals.

The specification discloses only the sequence of a single human proinsulin protein, and does not disclose any human cDNA sequence at all.

In addition, there is no evidence on the record of a relationship between the structure of rat insulin cDNA and the structure of insulin cDNAs from humans or other mammals that would provide any reliable information about the structure of other insulin cDNAs on the basis of the rat insulin cDNA.

There is no evidence on the record that the disclosed rat cDNA proinsulin sequence had a known structural relationship to the human cDNA sequence, or to other mammalian cDNA sequences; the specification discloses only a single human proinsulin (protein) sequence; the art indicated that human proinsulin proteins were expected to be variable in structure; and there is expected to be variation among human cDNAs that

encode a given human proinsulin. In view of the these considerations, a person of skill in the art would not have viewed the teachings of the specification as sufficient to show that the applicant was in possession of the claimed human cDNA.

Claim 1:

Claim 1 is directed to a genus of mammalian cDNAs which encode insulin. The specification evidences actual reduction to practice of the rat cDNA sequences for proinsulin and preproinsulin, but does not disclose any other cDNA sequences. The art indicates that there is likely to be substantial variation among the species within the genus of cDNAs that encode mammalian insulins because the sequences of the mammalian insulin proteins, and therefore the mammalian cDNAs, would be expected to vary among species.

The specification discloses a method for determining the corresponding human and other mammalian insulin cDNA sequences as well as the function of the claimed sequences. However, neither the specification nor the general knowledge of those skilled in the art provide evidence of any partial structure which would be expected to be common to the members of the genus. Moreover, there is post filing date evidence that indicates that there is a lack of a structural relationship between the rat insulin cDNA sequences and other mammalian insulin cDNA sequences. In view of the above considerations one of skill in the art would not recognize that applicant was in possession of the necessary common features or attributes possessed by members of the genus, because rat cDNA sequences are not representative of the claimed genus. Consequently, since applicant was in

possession only of the rat insulin cDNA and since the art recognized variation among the species of the genus of cDNAs that encode mammalian insulin, the rat insulin cDNA was not representative of the claimed genus. Therefore, the applicant was not in possession of the genus of mammalian insulin cDNAs as encompassed by claim 1.

Conclusion:

Claims 1 and 2 do not meet the written description requirement.

Example 18: <u>Process claim where the novelty is in the method steps.</u>

Specification: The specification teaches a method for producing proteins using mitochondria from the fungus *Neurospora crassa*. In the method, mitochondria are isolated from this fungus and transformed with a mitochondrial expression vector which comprises a nucleic acid encoding a protein of interest. The protein is subsequently expressed, the mitochondria is lysed, and the protein is isolated. The specification exemplifies the expression of β-galactosidase using the claimed method using a cytochrome oxidase promoter.

Claim:

1. A method of producing a protein of interest comprising;

obtaining *Neurospora crassa* mitochondria,

transforming said mitochondria with a expression vector comprising a nucleic acid that encodes said protein of interest,

expressing said protein in said mitochondria, and

recovering said protein of interest.

Analysis:

A review of the specification reveals that *Neurospora crassa* mitochondrial gene expression is essential to the function/operation of the claimed invention. A particular nucleic acid is not essential to the claimed invention.

A search of the prior art reveals that the claimed method of expression in *Neurospora crassa* is novel and unobvious.

The claim is drawn to a genus, i.e., any of a variety of methods that can be used for expressing protein in the mitochondria.

There is actual reduction to practice of a single embodiment, i.e., the expression of β-galactosidase.

The art indicates that there is no substantial variation within the genus because there are a limited number of ways to practice the process steps of the claimed invention.

The single embodiment is representative of the genus based on the disclosure of *Neurospora crassa* mitochondria as a gene expression system, considered along with the level of skill and knowledge in the gene expression art. One of skill in the art would recognize that applicant was in possession of all of the various expression methods necessary to practice the claimed invention.

Conclusion:

The claimed invention is adequately described.